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PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Assistant Commissioner for Patents
United States Patent and Trademark Office
Box PCT
Washington, D.C. 20231
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 20 January 2000 (20.01.00)	
International application No. PCT/NL99/00285	Applicant's or agent's file reference P21797PC00
International filing date (day/month/year) 07 May 1999 (07.05.99)	Priority date (day/month/year) 08 May 1998 (08.05.98)

Applicant

VOORBERG, Johannes, Jacobus et al

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

07 December 1999 (07.12.99)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland	Authorized officer R. E. Stoffel
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38

PATENT COOPERATION TREATY

PCT

NOTIFICATION OF THE RECORDING OF A CHANGE

(PCT Rule 92bis.1 and Administrative Instructions, Section 422)

From the INTERNATIONAL BUREAU

To:

OTTEVANGERS, S., U.
Vereenigde
Nieuwe Parklaan 97
NL-2587 BN The Hague
PAYS-BAS

Date of mailing (day/month/year)
18 April 2000 (18.04.00)

Applicant's or agent's file reference
P21797PC00

IMPORTANT NOTIFICATION

International application No.
PCT/NL99/00285

International filing date (day/month/year)
07 May 1999 (07.05.99)

1. The following indications appeared on record concerning:

[ ] the applicant [ ] the inventor [X] the agent [ ] the common representative

Name and Address
OTTEVANGERS, S., U.
Vereenigde Octrooibureaux
Nieuwe Parklaan 97
NL-2587 BN The Hague
Netherlands

State of Nationality:
State of Residence:
Telephone No.: 070 4166 711
Facsimile No.: 070 4166 799
Teleprinter No.:

2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning:

[ ] the person [ ] the name [X] the address [ ] the nationality [ ] the residence

Name and Address
OTTEVANGERS, S., U.
Vereenigde
Nieuwe Parklaan 97
NL-2587 BN The Hague
Netherlands

State of Nationality:
State of Residence:
Telephone No.: 070 4166 711
Facsimile No.: 070 4166 799
Teleprinter No.:

3. Further observations, if necessary:
Please note that the company's name has changed

4. A copy of this notification has been sent to:

[X] the receiving Office
[ ] the International Searching Authority
[X] the International Preliminary Examining Authority
[ ] the designated Offices concerned
[X] the elected Offices concerned
[ ] other:

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland
Facsimile No.: (41-22) 740.14.35

Authorized officer
Beatriz Morariu
Telephone No.: (41-22) 338.83.38

PATENT COOPERATION TREATY

LN

by fax and post

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:

OTTEVANGERS, S. U.
VEREENIGDE
Nieuwe Parklaan 97
NL-2587 BN The Hague
PAYS-BAS

NRFz 8-11-2000

070-4166799

Kopie v/naar

TERMIJN
~~15 AUG. 2000~~

Beantwoord vóór:

bericht gezonden aan

def.

MAP

Applicant's or agent's file reference
P21797PC00

PCT

PTO/PCT Rec'd 06 NOV 2000

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL PRELIMINARY EXAMINATION REPORT
(PCT Rule 71.1)

Date of mailing *(day/month/year)*
03.08.00

IMPORTANT NOTIFICATION

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/NL99/00285	07/05/1999	08/05/1998

Applicant
STICHTING SANQUIN BLOEDVOORZIENING et al.

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.

2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.

3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. **REMINDER**

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices) (Article 39(1)) (see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/	Authorized officer
European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Büchler, S Tel.+49 89 2399-8090

Form PCT/IPEA/416 (July 1992)

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
P21797PC00		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/NL99/00285	07/05/1999	08/05/1998
International Patent Classification (IPC) or national classification and IPC C12N15/13		
Applicant STICHTING SANQUIN BLOEDVOORZIENING et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 7 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 3 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☒ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
07/12/1999	13.08.00
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Giebeler, K Telephone No. +49 89 2399 8546

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/NL99/00285

I. Basis of the report

1. This report has been drawn on the basis of (*substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.*):

Description, pages:

1-37	as originally filed				

Claims, No.:

1-23	as received on	07/06/2000	with letter of	07/06/2000	

Drawings, sheets:

1/20-20/20	as originally filed

2. The amendments have resulted in the cancellation of:

☐ the description, pages:
☐ the claims, Nos.:
☐ the drawings, sheets:

3. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect of:

☐ the entire international application.

☒ claims Nos. 1-8, 14-16 (all partially), 9-12 (totally).

because:

☐ the said international application, or the said claims Nos. relate to the following subject matter which does not require an international preliminary examination (*specify*):

☒ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. 1-12, 14-16 are so unclear that no meaningful opinion could be formed (*specify*):

see separate sheet

☒ the claims, or said claims Nos. 1-12, 14-16 are so inadequately supported by the description that no meaningful opinion could be formed.

☐ no international search report has been established for the said claims Nos. .

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-8, 19
	No:	Claims	13-18, 20-23
Inventive step (IS)	Yes:	Claims	
	No:	Claims	1-8, 19
Industrial applicability (IA)	Yes:	Claims	1-8, 13-15, 17-21
	No:	Claims	16, 22, 23

2. Citations and explanations

see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

see separate sheet

Re Item III

Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

1. The subject-matter of claim 1(c) is too unclear to be examined since it defines a polynucleotide merely by its capability of hybridizing to a polynucleotide according to (a) or (b) whose sequence is also not defined. It is therefore impossible for the reader to determine the scope of the claim. Even nucleotide triplets or other known polynucleotides could be encompassed by said claim.
It should be noted that already claims (a) and (b) are unclear due to the lack of technical features, i.e. the nucleotide sequence of the claimed polynucleotides, but in the case of claim 1(c) not even a functional limitation is given.
Claim 9 is also too unclear to be examined since the sequence of the claimed polypeptide is only defined by the unclear expression "corresponding to or mimicking a fragment or derivative of a human antibody...". Fragments can be as small as one or two amino acids and the terms "mimicking" and "derivative" are so vague and unclear that they have no limiting effect whatsoever. Again, it is impossible for the reader to determine the scope of the claim. Consequently, claims referring to claim 9 could also not be examined.
Claims 1(c) and 9 and claims referring thereto are furthermore neither supported by the description nor sufficiently disclosed.

   Therefore, the present opinion has only been established for claim 1(a)(b), claims 2-8 only insofar they refer to claim 1(a)(b), claim 13, claims 14-16 only insofar they refer to claim 13, and claims 17-23.

Re Item V

Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

2. The following documents are cited:

   D1: JOURNAL OF MOLECULAR BIOLOGY, vol. 222, 1991, pages 581-597
   D2: WO 93 12232 A

D3: WO 93 03151 A
D4: EP-A-0 659 766
D5: WO 96 169745 A
D6: BLOOD, vol. 82, no. 8, October 1993 (1993-10), pages 2452-2461
D7: EP-A-0 152 746
D8: US-A-5 543 145
D9: BLOOD COAGULATION AND FIBROLYSIS, vol. 8, 1997, pages S15-S18
D10: WO 96 05860 A
D11: BLOOD, vol. 91, no. 7, 1998, pages 2347-235
D12: AMERICAN JOURNAL OF MEDICINE, vol. 91, 1991, pages 405-445

3. The present application does not satisfy the criterion set forth in Article 33(1)(2) PCT because the subject-matter of claims 13-18 and 20-23 is not new.

Antibodies (i.e. polypeptides) with factor VIII specificity and anti-idiotypic antibodies directed against a human antibody with factor VIII specificity have been known from the art, see for instance each of the documents D6, D7 and D8. These antibodies have also been suggested for medical use.
The term "recombinant" refers to the process of production of the claimed product and cannot establish novelty since a recombinantly produced antibody does not necessarily differ from one that has been obtained conventionally.
As concerns claim 17, the Applicant's argument that the capability of the claimed polypeptide to interfere with the activity of factor VIII inhibitors excludes the antibody per se could no be followed, because an antibody can also interfere with the activity of factor VIII inhibitors by competitive binding.
The unclear term "essentially" used in claim 18 can also not be used to distinguish the claimed matter from the prior art.
Therefore, claims 13-18 and 20-23 lack novelty.

4. The present application does not satisfy the criterion set forth in Article 33(3) PCT because the subject-matter of claims 1(a)(b), 2-8 and claim 19 referring thereto does not involve an inventive step.

The technical problem underlying the present application is the provision of polynucleotides coding for (known) human antibodies with factor VIII specificity.

However, methods of producing polynucleotides coding for antibodies with known specificity were well-known and standard at the priority date of the application, see for instance any of the documents D1, D2, D3, D4 and D5.
Therefore, it would have been obvious to provide the subject-matter of claims 1(a)(b), 2-8 and 19.

5. It should however be noted that a polypeptide capable of specific binding to the C2-domain of factor VIII and interference with the activity of factor VIII inhibitory antibodies, which polypeptide does not interfere with the factor VIII biological activity and comprises the variable part of the heavy chain of a human antibody with factor VIII specificity would appear to be novel over the available prior art and, moreover involve an inventive step. It appears that such polypeptides would not have been obvious to a person skilled in the art faced with the problem of providing compounds useful to block the action of inhibitory antibodies.

6. For the assessment of the present claims 16, 22 and 23 on the question whether it is industrially applicable, no unified criteria exist in the PCT Contracting States. The patentability can also be dependent upon the formulation of the claims. The EPO, for example, does not recognize as industrially applicable the subject-matter of claims to the use of a compound in medical treatment, but may allow, however, claims to a known compound for first use in medical treatment and the use of such a compound for the manufacture of a medicament for a new medical treatment.

Re Item VIII
Certain observations on the international application

7. The application does not meet the requirements of Article 6 PCT because the claims are not clear.

Claims 1(c) and 9 are completely unclear, see Item III above for reasons.

Claim 1(a)(b) is unclear since it lacks technical features, e.g. the sequence of the claimed polynucleotides.

Claim 2 is confusing and unclear since polynucleotides coding for an antibody with factor VIII specificity are much longer than 8 or 10 nucleotides.

Claims 3, 5, 8 and 16 are unclear due to the use of the term "optionally", which introduces ambiguity since it has no limiting effect on the scope of the claim. Preferred embodiments should be formulated as dependent claims covering features which are merely optional (Rule 6.4 PCT).

Claims 5, 6 and 7 are ambiguous and unclear because of the term "complement of the" in brackets.

Claim 18 is unclear due to the use of the term "essentially".

8. Independent claim 17 does not meet the requirement following from Article 6 PCT taken in combination with Rule 6.3(b) PCT that any independent claim must contain all the technical features essential to the definition of the invention, since it does not state that the claimed polypeptides do not interfere with factor VIII biological activity. Furthermore, it is not stated in the claim that the polypeptide is capable of specific binding to the **C2-domain** of factor VIII, and the claimed polypeptides which bind to other regions of factor VIII are neither supported by the description (Article 6 PCT), nor enabled by the application (Article 5 PCT).

PATENT COOPERATION TREAT

From the INTERNATIONAL SEARCHING AUTHORITY

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL SEARCH REPORT OR THE DECLARATION

(PCT Rule 44.1)

To:
VEREENIGDE OCTROOIBUREAUX
Attn. OTTEVANGERS, S. U.
Nieuwe Parklaan 97
NL-2587 BN The Hague
NETHERLANDS

Date of mailing *(day/month/year)*	25/10/1999
Applicant's or agent's file reference: P21797PC00	**FOR FURTHER ACTION** See paragraphs 1 and 4 below
International application No.: PCT/NL 99/ 00285	International filing date *(day/month/year)* 07/05/1999
Applicant: STICHTING SANQUIN BLOEDVOORZIENING et al.	

1. [X] The applicant is hereby notified that the International Search Report has been established and is transmitted herewith.

Filing of amendments and statement under Article 19:
The applicant is entitled, if he so wishes, to amend the claims of the International Application (see Rule 46):

When? The time limit for filing such amendments is normally 2 months from the date of transmittal of the International Search Report; however, for more details, see the notes on the accompanying sheet.

Where? Directly to the International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland
Fascimile No.: (41-22) 740.14.35

For more detailed instructions, see the notes on the accompanying sheet.

2. [ ] The applicant is hereby notified that no International Search Report will be established and that the declaration under Article 17(2)(a) to that effect is transmitted herewith.

3. [ ] **With regard to the protest** against payment of (an) additional fee(s) under Rule 40.2, the applicant is notified that:

[ ] the protest together with the decision thereon has been transmitted to the International Bureau together with the applicant's request to forward the texts of both the protest and the decision thereon to the designated Offices.

[ ] no decision has been made yet on the protest; the applicant will be notified as soon as a decision is made.

4. **Further action(s):** The applicant is reminded of the following:

Shortly after **18 months** from the priority date, the international application will be published by the International Bureau. If the applicant wishes to avoid or postpone publication, a notice of withdrawal of the international application, or of the priority claim, must reach the International Bureau as provided in Rules 90*bis*.1 and 90*bis*.3, respectively, before the completion of the technical preparations for international publication.

Within **19 months** from the priority date, a demand for international preliminary examination must be filed if the applicant wishes to postpone the entry into the national phase until 30 months from the priority date (in some Offices even later).

Within **20 months** from the priority date, the applicant must perform the prescribed acts for entry into the national phase before all designated Offices which have not been elected in the demand or in a later election within 19 months from the priority date or could not be elected because they are not bound by Chapter II.

Name and mailing address of the International Searching Authority	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL-2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Barbara Klaver

Form PCT/ISA/220 (July 1998)

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	**FOR FURTHER ACTION** see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
P21797PC00		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/NL 99/00285	07/05/1999	08/05/1998
Applicant		
STICHTING SANQUIN BLOEDVOORZIENING et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 4 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

a. With regard to the **language**, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

[ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

[ ] contained in the international application in written form.

[ ] filed together with the international application in computer readable form.

[X] furnished subsequently to this Authority in written form.

[X] furnished subsequently to this Authority in computer readble form.

[X] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

[X] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [X] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title,**

[ ] the text is approved as submitted by the applicant.

[X] the text has been established by this Authority to read as follows:

METHOD FOR DIAGNOSIS AND TREATMENT OF HAEMOPHILIA A PATIENTS WITH AN INHIBITOR

5. With regard to the **abstract,**

[X] the text is approved as submitted by the applicant.

[ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. _____

[ ] as suggested by the applicant.

[ ] because the applicant failed to suggest a figure.

[ ] because this figure better characterizes the invention.

[X] None of the figures.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/NL 99/00285

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claim 16 is directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No
[redacted]/NL 99/00285

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/13 C07K16/36 C07K16/42 A61K39/395 A61K38/37
C12Q1/68 //(A61K39/395,38:37)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	MARKS J D ET AL: "BY-PASSING IMMUNIZATION HUMAN ANTIBODIES FROM V-GENE LIBRARIES DISPLAYED ON PHAGE" JOURNAL OF MOLECULAR BIOLOGY, vol. 222, 1991, pages 581-597, XP000670314 cited in the application the whole document ---	1-16
Y	WO 93 12232 A (DANA FARBER CANCER INST INC ;NEW ENGLAND DEACONNESS HOSPITA (US)) 24 June 1993 (1993-06-24) the whole document ---	1-16
Y	WO 93 03151 A (MEDICAL RES COUNCIL) 18 February 1993 (1993-02-18) the whole document --- -/--	1-16

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
8 October 1999	25/10/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Hagenmaier, S

1

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
[redacted]/NL 99/00285

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
Y	EP 0 659 766 A (SCHERING PLOUGH CORP) 28 June 1995 (1995-06-28) the whole document	1-16
Y	WO 96 16974 A (ORAVAX INC) 6 June 1996 (1996-06-06) the whole document	1-16
Y	GILLES ET AL.: "ANTI-FACTOR VIII ANTIBODIES OF HEMOPHILIAC PATIENTS ARE FREQUENTLY DIRECTED TOWARDS NONFUNCTIONAL DETERMINANTS AND DO NOT EXHIBIT ISOTYPIC RESTRICTION" BLOOD, vol. 82, no. 8, October 1993 (1993-10), pages 2452-2461, XP002096815 the whole document	1-16
Y	EP 0 152 746 A (CHIRON CORP ;NORDISK GENTOFTE (DK)) 28 August 1985 (1985-08-28) the whole document	1-16
Y	US 5 543 145 A (SAINT-REMY JEAN-MARIE ET AL) 6 August 1996 (1996-08-06) the whole document	1-16
A	SULTAN: "ACQUIRED HEMOPHILIA AND ITS TREATMENT" BLOOD COAGULATION AND FIBROLYSIS, vol. 8, 1997, pages S15-S18, XP002096816 the whole document	
A	WO 96 05860 A (NOVONORDISK AS) 29 February 1996 (1996-02-29) the whole document	
A	FIJNVANDRAAT ET AL.: "A HUMAN ALLOANTIBODY INTERFERES WITH BINDING OF FACTOR IXa TO THE FACTOR VIII LIGHT CHAIN" BLOOD, vol. 91, no. 7, 1998, pages 2347-2352, XP002118155 the whole document	
A	HOYER L W: "FUTURE APPROACHES TO FACTOR VIII INHIBITOR THERAPY" AMERICAN JOURNAL OF MEDICINE, vol. 91, no. 5A, 4 November 1991 (1991-11-04), pages 405-445, XP002912577 the whole document	

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
/NL 99/00285

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
WO 9312232	A	24-06-1993	AU	668374 B	02-05-1996
			AU	3325993 A	19-07-1993
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CLAIMS

1. A polynucleotide in substantially isolated form, comprising a contiguous nucleotide sequence (a) coding for a human antibody with factor VIII specificity, or (b) complementary to a nucleotide sequence coding for a human antibody with factor VIII specificity, or (c) capable of selectively hybridizing under stringent conditions to nucleotide sequence (a) or (b).

2. A polynucleotide according to claim 1, wherein said contiguous nucleotide sequence is at least 8, preferably at least 10 nucleotides.

3. A probe or primer which comprises a polynucleotide according to claim 1 or claim 2, optionally further comprising a detectable label, such as a radioactive atom or group, an enzyme, a fluorescent or luminescent group, a dye or biotin.

4. An assay kit for detecting nucleic acid coding for a human antibody with factor VIII specificity, comprising a probe or primer according to claim 3 in a suitable container.

5. A nucleic acid amplification and detection kit for detecting nucleic acid coding for a human antibody with factor VIII specificity, comprising a pair of primers according to claim 3 capable of priming the synthesis of cDNA, and optionally further comprising a probe according to claim 3 capable of selectively hybridizing to (the complement of) a region of the nucleic acid to be detected between and not including the sequences from which the primers are derived.

6. A method for assaying a sample for the presence or absence of nucleic acid coding for a human antibody with factor VIII specificity, comprising contacting the sample with a probe according to claim 3 under conditions that allow the selective hybridization of said probe to the (complement of the) nucleic acid to be detected in the sample, and determining whether polynucleotide duplexes comprising said probe are formed.

7. A method for assaying a sample for the presence or absence of nucleic acid coding for a human antibody with factor VIII specificity, comprising subjecting nucleic acid present in the sample to a nucleic acid amplification process using a pair of primers according to claim 3 capable of priming the synthesis of cDNA, contacting the nucleic acid resulting from the amplification process with a probe according to claim 3 under conditions that allow the selective hybridization of said probe to the (complement of the) nucleic acid to

be detected in the sample, and determining whether polynucleotide duplexes comprising said probe are formed.

8. A method of producing a recombinant polypeptide, comprising providing a polynucleotide coding for said polypeptide, preparing a recombinant vector containing said polynucleotide operably linked to a control sequence capable of providing for the expression of the polynucleotide by a host cell, transforming a host cell with said recombinant vector, growing said host cell under conditions that provide for the expression of the polynucleotide and optionally isolating the thus produced polypeptide, wherein said polynucleotide codes for a human antibody with factor VIII specificity, or a fragment or derivative thereof capable of specific binding to factor VIII.

9. A polypeptide in substantially isolated form, comprising a contiguous amino acid sequence corresponding to or mimicking a fragment or derivative of a human antibody with factor VIII specificity capable of specific binding to factor VIII.

10. A polypeptide according to claim 9, wherein said contiguous amino acid sequence is capable of reducing the activity of factor VIII inhibiting antibodies.

11. A polypeptide according to claim 9 or claim 10, wherein said fragment is (part of) a variable region of the heavy chain or light chain of said antibody.

12. A polypeptide according to claim 9 or claim 10, wherein said derivative is a single chain Fv fragment of said antibody.

13. An antibody in substantially isolated form, comprising a recombinant human antibody with factor VIII specificity or an anti-idiotypic antibody directed against a human antibody with factor VIII specificity.

14. A pharmaceutical composition for the treatment of factor VIII inhibition in a human individual, comprising a polypeptide according to any one of claims 9-12 or an antibody according to claim 13, together with a pharmaceutically acceptable carrier.

15. A composition according to claim 14, which further contains factor VIII or a substitute of factor VIII.

16. A method of treatment of factor VIII inhibition in a human individual, comprising administering to said individual a polypeptide according to any one of claims 9-12 or an antibody according to claim 13, optionally together with factor VIII or a substitute of factor VIII.

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(71) Applicant *(for all designated States except US)*: STICHTING SANQUIN BLOEDVOORZIENING [NL/NL]; Plesmanlaan 125, NL–1066 CX Amsterdam (NL).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: VOORBERG, Johannes, Jacobus [NL/NL]; Klokkemeet 22, NL–1566 RH Assendelft (NL). VAN DEN BRINK, Edward, Norbert [NL/NL]; Valkenisseweg 105, NL–1069 BX Amsterdam (NL). TURENHOUT, Ellen, Anne, Maria [NL/NL]; Lavendel 19, NL–2211 MV Noordwijkerhout (NL).

(74) Agent: OTTEVANGERS, S., U.; Vereenigde Octrooibureaux, Nieuwe Parklaan 97, NL–2587 BN The Hague (NL).

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(57) Abstract

A polynucleotide, comprising a contiguous nucleotide sequence coding for a human antibody with factor VIII specificity, or complementary to a nucleotide sequence coding for a human antibody with factor VIII specificity, or capable of selectively hybridizing under stringent conditions to such nucleotide sequence. Such polynucleotide may be used as a probe or primer for detection of factor VIII inhibitors, or be used for producing a recombinant polypeptide. A polypeptide, comprising a contiguous amino acid sequence corresponding to or mimicking a fragment or derivative of a human antibody with factor VIII specificity capable of specific binding to factor VIII. An antibody, comprising a recombinant human antibody with factor VIII specificity or an anti–idiotypic antibody directed against a human antibody with factor VIII specificity. Pharmaceutical compositions which contain such polypeptide or antibody.

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METHODS FOR DIAGNOSIS AND TREATMENT OF HAEMOPHILIA A PATIENTS WITH AN INHIBITOR

FIELD OF THE INVENTION

This invention is in the fields of diagnosis and medical treatment. More in particular, the invention provides means and methods for diagnosing the presence of inhibitory antibodies directed against factor VIII in the blood of human individuals, and provides means, pharmaceutical compositions and methods for treating human individuals in which such inhibitory antibodies occur.

BACKGROUND OF THE INVENTION

Haemophilia A is an X-linked bleeding disorder which is characterized by the functional absence of blood coagulation factor VIII. Depending on the residual factor VIII activity in the plasma of the patient, haemophilia A can be classified as severe (factor VIII < 1%), moderate (factor VIII 1-5%) or mild (>5%). Bleeding episodes in patients with haemophilia A can be effectively controlled by intravenous administration of purified factor VIII concentrates. These factor VIII-concentrates may be derived from pools of human plasma. Alternatively, recombinant factor VIII produced by genetically engineered eukaryotic cells may be used as a starting material for the preparation of factor VIII concentrates.

A serious complication of current haemophilia A treatment constitutes the development of neutralizing antibodies directed against factor VIII. These antibodies, commonly termed factor VIII inhibitors, arise in approximately 25% of the patients with severe haemophilia A, usually after 5-20 exposure-days (Ehrenforth et al. 1992, Lancet 339: 594-598). In patients with moderate and mild haemophilia A, anti-factor VIII antibodies occur less frequently and this is most likely due to induction of tolerance by endogenous factor VIII present in the plasma of this group of patients (McMillan et al. 1988, Blood 71: 344-348). Antibodies to factor VIII may develop with low frequency in healthy individuals.

Diagnosis of factor VIII inhibitors is commonly performed using the so-called Bethesda assay (Kasper et al. 1975, Thromb. Diath. Haemorrh. 34: 869-

872). In this assay equal amounts of normal plasma and dilutions of inhibitor plasma are incubated for two hours at 37°C. Next, residual factor VIII activity is determined and compared to control incubation in which normal plasma is incubated with 0.1 M imidazole for 2 hours at 37°C. The amount of inhibitor is expressed in Bethesda units; one Bethesda unit corresponds to the amount of inhibitor that is capable of reducing the activity of factor VIII in normal plasma with 50%. A recent study has proposed several adaptations to the original assay system which serve to improve the stability of factor VIII during the assay (Verbruggen et al. 1995, Thromb. Haemostas. 73: 247-251). This so-called "Nijmegen modification" of the Bethesda assay is particularly useful for the detection of low titre factor VIII inhibitors. It should be noted that the Bethesda assay does not provide information on the epitopes of factor VIII inhibitory antibodies.

The occurrence of factor VIII-inhibiting antibodies renders factor VIII replacement therapy inadequate. Several treatment options are available to the clinician. Low titre inhibitors (up to 5-10 BU/ml) are usually treated with infusion of high doses of factor VIII. A subset of factor VIII inhibitors does not cross react with porcine factor VIII. Porcine factor VIII has been used for management of patients with an inhibitor. Administration of porcine factor VIII may present with side effects. After multiple treatment 30-50% of the patients develop antibodies that neutralize the activity of the administered porcine factor VIII.

An alternative treatment of patients with factor VIII inhibitor constitutes the use of factor VIII bypassing agents. Activated prothrombin concentrates complexes (APCC) have been used to bypass the activity of factor VIII. APCC has been used successfully to control bleeding episodes in a large number of patients with an inhibitor. However, treatment is not effective in all cases and an anamnestic rise in the titre of the inhibitor following administration of APCC (most likely due to trace amounts of factor VIII in the preparation) has been reported in a number of patients. In the last 5 years recombinant factor VIIa has become available as a new factor VIII bypassing agent for the treatment of patients with an inhibitor (Lusher et al. 1996. Haemostasis 26 (suppl. 1): 124-130). Recombinant factor VIIa has been successfully used to control the bleeding episodes in patients with an inhibitor. Treatment by this agent is however limited by the short half-life of this compound in the circulation which requires multiple infusions at relatively short time intervals. APC-resistant factor V has recently

been suggested as an alternative means to bypass the biological activity of factor VIII inhibitors (WO 95/29259). The agents described above do not act directly on factor VIII inhibitors but merely serve to bypass factor VIII by infusion of large amounts of clotting factor concentrates with increased procoagulant activity.

Other methods of inhibitor neutralization have been proposed but their effectiveness has not been convincingly shown. Immunoglobulin preparations derived from plasma of healthy donors has been proposed as an active suppressor of factor VIII inhibitors (Sultan et al. 1984, Lancet 333, 765-768). Despite success in patients with acquired haemophilia A and high titre inhibitors, immunoglobulin preparations are not applied universally for treatment of patients with an inhibitor. The beneficial effects of immunoglobulin preparations in these patients have been attributed to the presence of anti-idiotypic antibodies that neutralize the activity of factor VIII inhibitors. Indeed in some patients the decline in the level of factor VIII inhibitors coincided with the appearance of anti-idiotypic antibodies (Sultan et al. 1987, Proc. Natl. Acad. Sci. USA 84: 828-831). Control of factor VIII inhibitors by anti-idiotypic antibodies in both haemophilia A patients and healthy individuals has been strongly advocated by some investigators (Gilles et al. 1996, J. Clin. Inv. 97: 1382-1388). The same group has proposed that infusion of antigen-antibody complexes in patients with inhibitors may accelerate a decline in anti-factor VIII antibodies in patients with an inhibitor (USP 5,543,145). It has been proposed that this decline is mediated by an increase in the number of anti-idiotypic antibodies which are induced by the infused antigen-antibody complexes. The factor VIII specific antibody used in this treatment protocol is derived from plasma of patients with an inhibitor. Obviously, this presents a heterogeneous mixture of antibodies and no details with respect to the epitope specificity of these antibodies are available. Also the primary structure of the antibodies in these antigen-antibody preparations has not been disclosed.

SUMMARY OF THE INVENTION

This invention relates to methods for diagnosis and treatment of inhibitory antibodies directed against factor VIII. Methods are disclosed that show how to arrive at nucleotide and amino acid sequences that encode factor VIII specific antibodies. This invention discloses diagnostic tests that allow for detection of nucleotide and amino acid sequences that encode factor VIII specific antibodies

within a heterogeneous mixture of antibody-encoding nucleotide or amino acid sequences. This invention further discloses how to use recombinant antibody fragments which bind specifically to factor VIII as novel therapeutic agents for the treatment of patients with factor VIII inhibitors.

The invention provides a polynucleotide in substantially isolated form, comprising a contiguous nucleotide sequence (a) coding for a human antibody with factor VIII specificity, or (b) complementary to a nucleotide sequence coding for a human antibody with factor VIII specificity, or (c) capable of selectively hybridizing under stringent conditions to nucleotide sequence (a) or (b).

Preferably, the contiguous nucleotide sequence is at least 8, preferably at least 10 nucleotides.

In a preferred embodiment, the invention provides a probe or primer which comprises a polynucleotide as defined herein, optionally further comprising a detectable label, such as a radioactive atom or group, an enzyme, a fluorescent or luminescent group, a dye or biotin.

The invention also provides an assay kit for detecting nucleic acid coding for a human antibody with factor VIII specificity, comprising a probe or primer as defined herein in a suitable container.

Furthermore, the invention provides a nucleic acid amplification and detection kit for detecting nucleic acid coding for a human antibody with factor VIII specificity, comprising a pair of primers as defined herein capable of priming the synthesis of cDNA, and optionally further comprising a probe as defined herein capable of selectively hybridizing to (the complement of) a region of the nucleic acid to be detected between and not including the sequences from which the primers are derived.

The invention provides a method for assaying a sample for the presence or absence of nucleic acid coding for a human antibody with factor VIII specificity, comprising contacting the sample with a probe as defined herein under conditions that allow the selective hybridization of said probe to the (complement of the) nucleic acid to be detected in the sample, and determining whether polynucleotide duplexes comprising said probe are formed.

The invention also provides a method for assaying a sample for the presence or absence of nucleic acid coding for a human antibody with factor VIII specificity, comprising subjecting nucleic acid present in the sample to a nucleic acid amplification process using a pair of primers as defined herein capable of priming the synthesis of cDNA, contacting the nucleic acid resulting from the

amplification process with a probe as defined herein under conditions that allow the selective hybridization of said probe to the (complement of the) nucleic acid to be detected in the sample, and determining whether polynucleotide duplexes comprising said probe are formed.

Furthermore, the invention provides a method of producing a recombinant polypeptide, comprising providing a polynucleotide coding for said polypeptide, preparing a recombinant vector containing said polynucleotide operably linked to a control sequence capable of providing for the expression of the polynucleotide by a host cell, transforming a host cell with said recombinant vector, growing said host cell under conditions that provide for the expression of the polynucleotide and optionally isolating the thus produced polypeptide, wherein said polynucleotide codes for a human antibody with factor VIII specificity, or a fragment or derivative thereof capable of specific binding to factor VIII.

According to another aspect, the invention provides a polypeptide in substantially isolated form, comprising a contiguous amino acid sequence corresponding to or mimicking a fragment or derivative of a human antibody with factor VIII specificity capable of specific binding to factor VIII. In a preferred embodiment of the invention, the contiguous amino acid sequence is capable of reducing the activity of factor VIII inhibiting antibodies.

Preferably, the fragment is (part of) a variable region of the heavy chain or light chain of said antibody, and the derivative is preferably a single chain Fv fragment of said antibody.

The invention furthermore provides an antibody in substantially isolated form, comprising a recombinant human antibody with factor VIII specificity or an anti-idiotypic antibody directed against a human antibody with factor VIII specificity.

The invention furthermore provides a pharmaceutical composition for the treatment of factor VIII inhibition in a human individual, comprising a polypeptide as defined herein or an antibody as defined herein, together with a pharmaceutically acceptable carrier. Optionally, the composition further contains factor VIII, or a substitute of factor VIII.

The invention also provides a method of treatment of factor VIII inhibition in a human individual comprising administering (an effective amount to reduce or prevent said factor VIII inhibition of) a polypeptide as defined herein or an antibody as defined herein, optionally together with factor VIII or a substitute of factor VIII.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the light chain specificity of 12 clones obtained after 4 rounds of panning of the IgG4-specific library described in Example 2. Phage expressing recombinant antibodies were incubated on microtiter wells which contained factor VIII light chain (black bars; +80K). To correct for background binding phage were also incubated on microtiter wells that did not contain factor VIII light chain (grey bars; -80K). On the Y-axis the OD(450-540 nm) is depicted. Two clones (c) express antibody fragments that do not bind specifically to the factor VIII light chain.

Figure 2 shows the light chain specificity of 12 clones randomly chosen after the first round of panning of the IgG4-specific library described in Example 2. Clones 7, 8 and 10 express antibody fragments with factor VIII light chain specificity. The other clones do not specifically bind to the factor VIII light chain. Only background binding of the phage to the microtiter wells is observed (grey bars). Two clones (c) express antibody fragments that do not bind specifically to the factor VIII light chain.

Figure 3 shows the nucleotide sequence of clone EL14 and clone IT2. The nucleotide sequence of both clones is aligned with the nucleotide sequence of the germline sequences DP-10 (for EL14) and DP-14 (for IT2). The different regions of the variable part of the heavy chain are indicated in the following order: framework 1, CDR1, framework 2, CDR2, framework 3, CDR3 and framework 4. Homology of clones EL14 and IT2 with the germline sequences DP-10 and DP-14 is indicated by horizontal bars (-). Differences are indicated by the nucleotides that occur in the germline sequences DP-10 and DP-14. Note that both CDR3 and framework 4 are not derived from the germline sequences DP-10 and DP-14. Consequently, no homology is given for this part of the nucleotide sequence.

Figure 4A gives the amino acid sequence derived of the nucleotide sequence of clone EL14 and IT2. Deviations in the amino acid sequence of the germline segments DP-10 and DP-14 are indicated in the lower lines. Framework is abbreviated as "FR".

Figure 4B compares the amino acid sequence of three related clones that are derived from the germline segment DP-14. The amino acid sequences of clone IT2, clone EL5 and clone EL25 are compared to that of the germline segment DP-14. Deviations in amino acid sequence are indicated for each clone. Note that some amino acid substitutions are shared by the three different clones.

Figure 4C compares the amino acid sequences of the third variable loop (CDR3) of the heavy chain of clone EL14 and IT2. Homologous amino acid residues are indicated by vertical lines. Dots denote amino acids related in charge or hydrophobicity.

Figure 5 shows the specificity of binding of scFv-EL14 and scFv-IT2 to the factor VIII light chain as assessed by the murine monoclonal antibodies CLB-CAg A and CLB-CAg 117. ScFv-EL14 binds specifically to the factor VIII light chain when peroxidase labelled CLB-CAg A (80K/Apo) is used as an indicator antibody (hatched bars). Also scFv-IT2 binds to the factor VIII light chain under these conditions (hatched bars). In contrast, when peroxidase labelled CLB-CAg 117 (80K/117po) is used as indicator antibody binding of scFv-EL14 and scFv-IT2 is strongly reduced (black bars). No binding is observed in the absence of factor VIII light chain (-/117po; -/Apo). Clone O4 does not bind to factor VIII under these experimental conditions. These experiments show that the epitope of scFv-EL14 and scFv-IT2 overlaps with that of CLB-CAg 117. On the y-axis the absorbance OD (450-540 nm) is given. On the x-axis scFv-EL14, scFv-IT2 and scFv-O4 are given.

Figure 6 shows the binding of different dilutions of purified scFv-EL14 (open circles), scFv-IT2 (closed circles) and scFv-O4 (negative control). On the x-axis the different concentrations of protein tested are indicated (μg/ml), on the y-axis the absorbance OD(450-540 nm) is given. Clone scFv-O4 does not bind to the factor VIII light chain at the protein concentration tested in this experiment. Both scFv-IT2 and scFv-EL14 bind to the factor VIII light chain. ScFv-EL14 binds with a higher affinity to the factor VIII light chain when compared to scFv-IT2.

Figure 7A shows the neutralization of the inhibitory activity of the murine monoclonal antibody CLB-CAg 117 by scFv-EL14. Antibody CLB-CAg 117 was diluted till a value of 2 BU/ml which corresponds with a residual factor VIII activity of about 25%. Increasing amounts of scFv-EL14 were capable of neutralizing the inhibitory activity of CLB-CAg 117 (closed circles). A concentration of 0.75 μg/ml suffices to restore factor VIII activity to its original level. ScFv-EL14 did not affect the inhibitory activity of the murine monoclonal antibody CLB-CAg A (open circles). On the y-axis residual factor VIII activity is depicted. On the x-axis the amount of scFv added is given in μg/ml.

Figure 7B shows the neutralization of the inhibitory activity of CLB-CAg 117 by scFv-IT2 (closed circles). A concentration of 65 μg/ml is needed to

restore factor VIII activity to its original level. ScFv-IT2 did not affect the inhibitory activity of CLB-CAg A (open bars). On the y-axis residual factor VIII activity is depicted. On the x-axis the amount of scFv added is given in µg/ml.

Figure 8A shows the epitope specificity of 15 clones obtained after four rounds of panning of the IgG4-specific library described in example 8. Panning was performed using factor VIII immoblized on immunotubes. Phage expressing recombinant antibodies were incubated on microtiter wells which contained factor VIII light chain (black bars) or A3-C1-domain (hatched bars). To correct for background binding, phage were also incubated on microtiter wells that did not contain factor VIII light chain or A3-C1 domain (white bars). On the y-axis the OD (450-540 nm) is depicted.

Figure 8B shows the epitope specificity of 15 clones obtained after four rounds of panning the IgG4 specific library described in example 8. Panning was performed using factor VIII light chain that had been immoblized in microtiter wells employing CLB-CAg 117. Phage expressing recombinant antibodies were incubated on microtiter wells which contained factor VIII light chain (black bars) or A3-C1-domain (hatched bars). To correct for background binding, phage were also incubated on microtiter wells that did not contain factor VIII light chain or A3-C1 domain (white bars). On the y-axis the OD (450-540 nm) is depicted.

Figure 9A shows the deduced amino acid sequence of recombinant antibody fragments specific for the A3-C1 domain. The amino acid sequence of germ line variable heavy chain gene segments DP15, DP31, DP49 and DP77 is given. Deviations in amino acid sequence from these germline gene segments are indicated for clone B38, B18, B35 and B04. Also the amino acid of the CDR3 and FR4 of the A3-C1 specific recombinant antibodies encoded by clone B38, B18, B35 and B04 is given.

Figures 9B-E give the nucleotide and amino acid sequence of the variable heavy chain domain of clone B38, B18, B35 and B04.

Figure 10A shows the epitope specificity of 20 clones obtained after four rounds of panning of the IgG4-specific library described in Example 9. Panning was performed using factor VIII heavy chain (90K+) that had been immobilized in microtiter wells employing CLB-CAg 9. Phage expressing recombinant antibodies were incubated on microtiter wells which contained factor VIII heavy chain that was immobilized using the anti-heavy chain monoclonal antibody ESH5 (black bars). To correct for background binding,

phage were also incubated on microtiter wells that did not contain factor VIII heavy chain (white bars). On the y-axis the OD (450-540 nm) is depicted.

Figure 10B shows the epitope specificity of 20 clones obtained after four rounds of panning of the IgG4-specific library described in Example 9. Panning was performed using factor VIII heavy chain (90K+) that had been immobilized in immunotubes. Phage expressing recombinant antibodies were incubated on microtiter wells which contained factor VIII heavy chain that was immobilized using the anti-heavy chain monoclonal antibody ESH5 (black bars). To correct for background binding, phage were also incubated on microtiter wells that did not contain factor VIII heavy chain (white bars). On the y-axis the OD (450-540 nm) is depicted.

Figure 11A shows the deduced amino acid sequence of recombinant antibody fragments specific for the factor VIII heavy chain. The amino acid sequence of germ line variable heavy chain gene segments DP10 and DP47 is given. Deviations in amino acid sequence from these germline gene segments are indicated for two clones that encode recombinant antibodies that bind to the factor VIII heavy chain. Also the amino acid of the CDR3 and FR4 of the factor VIII heavy chain specific recombinant antibodies encoded by the two clones is given.

Figures 11B and C give the nucleotide and amino acid sequence of the variable heavy chain domain of two clones that encode recombinant antibodies that bind specifically to the factor VIII heavy chain.

DETAILED DESCRIPTION OF THE INVENTION

A number of investigators have addressed the epitope-specificity and mode of action of factor VIII inhibitory antibodies. Molecular cloning of the factor VIII cDNA revealed that factor VIII consists of a series of repeated domains which appear in the order A1-A2-B-A3-C1-C2. In plasma, factor VIII circulates as a heterodimer which consists of a heavy chain of variable length (90-220 kDa) and a light chain of 80 kDa. The factor VIII light chain consists of the domains A3-C1-C2 while the factor VIII heavy chain comprises the domains A1-A2-B. Heterogeneity of the factor VIII heavy chain is caused by limited proteolysis within the B-domain which contains several sites that are sensitive towards proteolytic cleavage. In plasma, factor VIII circulates in complex with von Willebrand factor, a large multimeric protein involved in the initial steps of platelet adhesion to a damaged vessel wall. Binding to von Willebrand factor protects factor VIII from proteolytic degradation. The physiological importance of this interaction is underscored by the low levels of factor VIII in plasma of patients that lack von Willebrand factor. Factor VIII is a precursor molecule which upon activation functions as a cofactor for factor IXa in the phospholipid and Ca^{2+}-dependent conversion of factor X to factor Xa. Activation of factor VIII involves proteolytic cleavages in both the heavy and light chain of factor VIII. Thrombin is considered to be the physiological activator of factor VIII and cleaves at Arg^{372}, Arg^{740} and Arg^{1689} of factor VIII. Thrombin activated factor VIII thus consists of a hetero-trimer of the separate A1 and A2-domains and the cleaved factor VIII light chain (A3-C1-C2). Cleavage at Arg^{1689} of the factor VIII light chain results in removal of amino-acid sequence Glu^{1649}-Arg^{1689} which is essential for binding of factor VIII to von Willebrand factor. Sofar, three major binding sites for factor VIII inhibitors have been characterized (Scandella et al. 1994, Blood 86: 1811-1819; Healey et al. 1995, J. Biol. Chem. 270: 14505-14509; Fijnvandraat et al. 1998, Blood 91: 2347-2352).

Amino acid residues Val^{2248}-Ser^{2312} in the C2-domain constitute a binding site for factor VIII inhibitors. The large size of this epitope suggests that a number of antibodies which bind to different amino acid regions in this area occur in plasma of patients with inhibitors of C2-specificity. The mechanisms of action of anti-C2 antibodies has been explored in considerable detail. Most of these antibodies interfere with binding of factor VIII to phospholipids. Furthermore, some of the antibodies with C2-specificity also inhibit the interaction of factor VIII with its carrier von Willebrand factor. A new

mechanism for inhibition of factor VIII by a human alloantibody has been described recently (Saenko et al. 1996, J. Biol. Chem. 271: 27424-27431). A human alloantibody that binds only to the amino-terminal portion (Val^{2248}-Gly^{2285}) of the C2-epitope has been shown to inhibit the thrombin induced release of factor VIII from von Willebrand factor.

Amino acid residues Arg^{484}-Ile^{508} in the A2-domain of factor VIII constitute a major epitope for factor VIII inhibitors. Studies on the mechanism of inhibition of anti-A2 antibodies have shown that anti-A2 antibodies interfere with conversion of factor X to Xa by the lipid bound factor VIIIa-factor IXa-complex (Lollar et al., 1995). The anti-A2-antibodies do not interfere with binding of factor X to the factor VIIIa-factor IXa complex but simply limit the conversion of factor X.

A third major epitope of factor VIII inhibitors has been found in the A3-domain of factor VIII. Binding of inhibitory antibodies was dependent on the presence of amino acids Gln^{1778}-Met^{1823}. Previous studies have shown that this site constitute a binding site for factor IXa and indeed antibodies binding to this site interfered with complex assembly of factor VIIIa and factor IXa (Fijnvandraat et al. 1998. Blood 91: 2347-2352). In a number of patients with an inhibitor, inhibitory antibodies directed against other epitopes have been observed. An early study has shown that inhibitory antibodies may recognize amino acid region Met^{336}-Arg^{372} of factor VIII (Ware et al. 1988. Proc. Natl. Acad. Sci USA 85: 3165-3169). The mechanism of inhibition has not yet been explored but recently a binding site for factor X has been proposed in this part of the factor VIII molecule (Lapan, K.A. and Fay, P.J. 1997. J. Biol. Chem. 272: 2082-2088).

The restricted epitope specificity of factor VIII inhibitors suggests that a limited number of dominant B-cell epitopes is involved in the immune response to factor VIII. Apparently, human anti-factor VIII antibodies synthesized by B-cell clones from a variety of patients are surprisingly similar with respect to epitope specificity. This suggests that the primary amino acid and nucleotide sequence of antibodies with factor VIII specificity is similar at the molecular level. Based on this it is desirable to define the presence and epitope specificity of anti-factor VIII antibodies by simply addressing the presence of nucleotide sequences that correspond to antibodies with factor VIII inhibiting capacity. Sofar, the primary sequences of anti-factor VIII antibodies have been poorly defined. Davies and co-workers have suggested an association between factor

VIII inhibitors and use of VH gene segment DP73 (Davies et al. 1997. Thromb. Haemostas. supplement: 2352A). The nucleotide and primary amino acid sequence of these antibodies has not been disclosed and details with respect to the epitope specificity of these antibodies are lacking. Clearly, there is a need to define the primary amino acid and nucleotide sequence of factor VIII antibodies in more detail. Such sequence information can be used to design diagnostic tests which can be used to monitor the occurrence of B-cell clones that produce factor VIII inhibitors in patients with haemophilia A. These diagnostic tests can be extremely sensitive and give information on the epitope specificity of factor VIII inhibitors.

Studies directed at defining the epitope specificity and mode of action of these antibodies are limited by the heterogeneity of these antibodies in the plasma of these patients. Clearly, more stringent diagnostic criteria would be required to define the properties of factor VIII inhibitors in more detail.

A sudden increase in the frequency of inhibitor development in a group of previously treated patients has been associated with a particular pasteurized factor VIII concentrate manufactured in the Netherlands (Roosendaal et al. 1993. Blood 81: 2180-2186). These factor VIII inhibitors are directed against the factor VIII light chain and epitope mapping revealed that the majority of inhibitors reacted with epitopes in the A3-C1 and the C2-domain of factor VIII (Sawamoto et al. 1998. Thromb. Haemostas. 79: 62-68). Recently, a second pasteurized factor VIII concentrate has been implicated in the development of inhibitors in a group of previously treated patients. Also in this case the inhibitory antibodies were predominantly of factor VIII light chain specificity (Peerlinck et al. 1997. Thromb. Haemostas. 77: 80-86). It has been suggested that inhibitor development in these patients is due to small alterations in the factor VIII molecule which have been induced by the manufacturing process. This may indicate that the antibodies that developed in these patients have different properties compared to the factor VIII inhibitory antibodies that develop in other patients. Clearly, knowledge of nucleotide and amino acid sequence of factor VIII specific antibodies could provide additional information on the etiology of factor VIII inhibitor which is desirable for the characterization of the antibody response in patients who have received these factor VIII concentrates.

Until now, the primary nucleotide and amino acid sequence of anti-factor VIII antibodies has not been disclosed. This invention describes the nucleotide sequences that encode human antibodies with factor VIII-specificity. Based on

the primary sequence of these antibodies, oligonucleotide primers are designed that allow for detection of B-cells that produce antibodies with affinity for factor VIII. Detection of factor VIII specific B-cells may be accomplished using both mRNA, cDNA or DNA which are derived from lymphocytes of patients. Genomic DNA, RNA and cDNA are prepared from lymphocytes by methods that are generally known in the art. Some methods for the detection of factor VIII specific B-cell clones are listed below. Other methods for the detection of nucleotide sequences of factor VIII specific antibodies, disclosed in this invention, are considered to fall within the scope of this invention. Selective amplification of heavy chain variable sequences (VH-genes) can be used to detect nucleotide sequences that encode antibodies that are part of the human antibody repertoire that can bind specifically to factor VIII. The variable part of the human heavy chain is assembled from the variable heavy chain regions (VH), the diversity regions (D) and the joining regions (J). Fusion of these three different gene segments is not a precise event and this so-called "junctional diversity", together with the process of nucleotide addition and deletion, results in the generation of the hypervariable complementary determining region 3 (CDR3). The human light chain is assembled in a similar manner but lacks diversity region D. Additional sequence diversity of both heavy and light chain sequences is generated by somatic hypermutation and together with the mechanisms outlined above this ultimately results in the generation of high affinity antibodies. Knowledge on the nucleotide sequences that encode factor VIII-specific antibody allows for the detection of this specific antibody in the repertoire of patients who are at risk of developing factor VIII-specific antibodies (such as haemophilia A patients who are treated with factor VIII or patients with acquired haemophilia). Amplification may be performed with a combination of oligonucleotide primers directed against constant regions or variable regions of heavy and light chain of factor VIII-specific antibodies. Detection of factor VIII specific antibodies may be performed using one oligonucleotide primer derived from the variable parts of the nucleotide sequences encoding factor VIII antibodies and one oligonucleotide primer that is derived from the constant regions of factor VIII specific antibodies. Detection may also be performed using two oligonucleotide primers specific for variable parts of the nucleotide sequence that encodes an antibody that binds to factor VIII. The methods described herein also include the amplification of immunoglobulin genes using oligonucleotide primers that are directed against the constant regions of the immunoglobulin

genes. Subsequent detection of nucleotide sequences of factor VIII specific antibodies can be performed using selective hybridization with (radiolabelled) oligonucleotide primers that are directed against the variable parts of the nucleotide sequence encoding factor VIII specific antibodies. From the above it follows that oligonucleotide primers are preferentially but not exclusively directed towards the constant and variable regions of factor VIII specific antibodies. In example 5, methods are disclosed that can be used to detect the presence of factor VIII specific antibodies in a mixture of nucleotide sequences. Combination of oligonucleotide primers derived from the nucleotide sequence of factor VIII specific antibodies can be used to directly assess the presence of factor VIII specific antibodies in the antibody-repertoire of patients. Alternatively, analysis by methods that include but are not limited to sequencing analysis, re-amplification of obtained fragments with more specific oligonucleotide primers, digestion with restriction enzymes and selective hybridization may be utilized to address the presence of factor VIII antibodies. Quantification of the amount of nucleotide sequences encoding factor VIII antibodies may be obtained by various methods that are generally known in the art and include but are not limited to the following. The amount of radioactivity incorporated into a PCR-fragment that encodes part of a factor VIII specific antibody can be determined. Furthermore, radioactively labelled oligonucleotide probes can be used to estimate the amount of a nucleotide sequence encoding a factor VIII specific antibody in a mixture of DNA fragments that code for part of a patients antibody repertoire. Quantitative PCR-amplification can be performed using for example dye-modified oligonucleotide primers which allow for direct monitoring of the amount of PCR-product generated during amplification.

Other methods that selectively detect and quantify specific nucleotide sequences that encode factor VIII specific antibodies may be devised by an average expert in the art. These methods are considered to fall within the scope of the present invention.

Examples 1-10 provide details on the identification and detection of nucleotide sequences that encode factor VIII specific antibodies in haemophilia patients. These examples teach how to arrive at the nucleotide sequence of factor VIII inhibitors and provide information on how to use this information for the detection of factor VIII specific antibodies.

This invention discloses the nucleotide and primary amino acid sequences of factor VIII specific antibodies. Factor VIII inhibitors are commonly directed

against three major epitopes on factor VIII within the A2- A3 and C2-domain of factor VIII. In Example 4 the nucleotide and amino acid sequence of anti-C2 antibodies is disclosed. In examples 8 and 9, the nucleotide and amino acid sequence of anti-A2 and anti-A3-C1 antibodies is disclosed. This invention teaches how to arrive at the nucleotide and amino acid sequence of factor VIII specific antibodies and the methods disclosed in this invention can be used to derive the nucleotide and amino acid sequence of anti-factor VIII antibodies with specificity for other domains of factor VIII which are a target for factor VIII inhibitors. Anti-factor VIII antibodies encoded by the nucleotide sequences disclosed here, can be used for the development of therapeutic agents that are capable of limiting the biological activity of factor VIII inhibitors. These therapeutic agents preferentially contain, but are not limited to:

1. The antibody fragments (or agents based on the nucleotide or amino acid sequence) can be used for the generation of anti-idiotypic antibodies. Antibody fragments can be either administered together with factor VIII or administered alone. Also, peptides or related agents which are based on the primary amino acid sequence of the variable parts of factor VIII specific antibodies can be used to induce the formation of anti-idiotypic antibodies direct against factor VIII inhibitors. Anti-idiotypic antibodies can also be obtained by screening large (semi-synthetic) libraries that encode a wide variety of recombinant antibodies. The preparation of anti-idiotypic antibodies can also take place in animals that include but are not limited to mouse by injection of recombinant antibody disclosed in this invention. Anti-idiotypic antibodies can subsequently be obtained by methods that are known to those skilled in the art. An anti-idiotypic response to factor VIII specific antibodies may also be obtained by injection of DNA encoding part of the nucleotide sequences of factor VIII specific antibodies which can be obtained by the methods outlined in this invention. Immunization by injection of DNA is considered to be only modestly immunogenic and other agents are needed to obtain a sufficiently high immune response. Co-injection of plasmid DNA encoding IL-2, GM-CSF and tetanus toxoid has been used to enhance the immune response to injected DNA (Spellerberg et al. 1997. J. Immunol. 15:1885-1892). Similar methods can be applied to enhance the immune response towards DNA fragments encoding factor VIII specific antibodies.

2. The recombinant antibody fragments described in this invention can be used as a therapeutic for treatment of patients with an inhibitor. Examples 7 and

10 disclose that recombinant antibody fragments (termed scFv's) which bind specifically to the C2-domain, interfere with binding of inhibitory antibodies to factor VIII. These scFv's can be used for treatment of patients with inhibitory antibodies directed against the C2-domain. This invention discloses how to arrive at recombinant antibody fragments that bind specifically to the A2, A3-C1 and C2-domain of factor VIII. Using the methods outlined in this invention additional antibody fragments directed against these and other regions on factor VIII may be obtained. These regions include but are not limited to Arg^{484}-Ile^{508} in the A2-domain, Gln^{1778}-Met^{1823} in the A3-domain and Val^{2248}-Ser^{2312} in the C2-domain. Recombinant antibodies directed against multiple epitopes preferentially will be part of a pharmaceutical preparation since most patients have inhibitory antibodies directed against multiple epitopes on factor VIII. The inhibitor neutralizing activity of the recombinant antibody fragments described in this invention may be modified by the introduction of point mutations in the constant and variable parts of these fragments. Furthermore, the recombinant antibody fragments described in this invention may be cloned into vectors which allow for expression of these fragments for example as Fab-fragment. Other vectors for expression of antibodies and antibody fragments are available to an average expert in the field (see for example: "Antibody Engineering; A Practical Approach" edited by Mc Cafferty et al. 1996. Oxford University Press). Methods to increase the affinity of recombinant antibodies or antibody fragments are readily available and can be used to modulate the biological activities of the recombinant antibody fragments described in this invention. Peptides and peptide-related agents which have been designed based on the amino acid sequence of the recombinant antibodies described in this invention (for example peptides derived from the amino acid sequence of CDR3) can be used to interfere with the activity of factor VIII inhibitors.

In summary, this invention provides an improved method to define factor VIII inhibitory antibodies. The methods described result in definition of the nucleotide sequence of factor VIII inhibitors and teaches how to utilize this information for the development of improved methods for the detection of factor VIII inhibitors. This invention also discloses pharmaceutical preparations, derived from the nucleotide and/or primary amino acid sequence of factor VIII specific antibodies, that can be used in the treatment of haemophilia A patients with inhibitory antibodies directed against factor VIII. The dose of the therapeutic agents to be administered to a patient, obviously depends on the

affinity of the therapeutic agent for factor VIII. The affinity of the recombinant antibody fragments described in this invention can vary considerably as is disclosed in Examples 6 and 7. The dose to be administered also depends on bodyweight of the patient, the titre of the factor VIII inhibitor and the biological activities of the different components of the therapeutic agent used. The dose to be administered can be estimated according to methods that are disclosed in Examples 7 and 10. These methods may be complemented with dose finding studies which involve administration of the therapeutic agents in animal models and healthy individuals. In general, the dose administered will vary between 10 µg - 5 g/kg and more preferably between 100 µg - 1g/kg of bodyweight per day.

The therapeutic agent may be administered in combination with factor VIII (or a substitute of factor VIII). The dose of factor VIII administered together with the therapeutic agent may vary between 0.1 and 2000 Units per kg and more preferably between 1 and 200 Units per kg of body weight per day.

The words "substantially isolated form" as used herein are intended to refer to a form of the material which is different from any naturally occurring form of said material, i.e. different from the material in its natural environment. In particular, the words are intended to define relative freedom or absence of substances that naturally accompany the material.

EXAMPLE 1: Characterization of anti-factor VIII antibodies in patient's plasma

Anti-factor VIII antibodies present in the plasma of a patient with acquired haemophilia were characterized by immunoprecipitation and neutralization experiments. The construction of recombinant factor VIII fragments corresponding to the A2, A3-C1-C2 and C2-domain of factor VIII has been described previously (Fijnvandraat et al. 1997. Blood 89: 4371-4377; Fijnvandraat et al. 1998. Blood 91: 2347-2352). These recombinant factor VIII fragments were metabolically labelled with [^{35}S]-methionine and subsequently used for the detection of anti-factor VIII antibodies by immunoprecipitation using methods that have been described previously (Fijnvandraat et al. 1998. Blood 91: 2347-2352). Reactivity with both metabolically labelled A2, A3-C1-C2 and C2 domain was observed (data not shown). This indicates that at least two classes of antibodies directed against factor VIII were present in the plasma of the patient. To determine the contribution of the different antibodies in the patient's plasma to the titre of the inhibitor as measured in the Bethesda assay we

performed neutralization experiments. Increasing concentrations of recombinant factor VIII fragments were mixed with samples that contained factor VIII antibodies diluted until a final inhibitory capacity of 2 BU/ml. Addition of both recombinant factor VIII light chain (A3-C1-C2) and C2-domain resulted in a decrease in the inhibitory activity of 50 and 20%, respectively. Addition of the factor VIII heavy chain (domains A1-A2-B) resulted in 45% neutralization of the inhibitor in the plasma of the patient. Based on these data we conclude that inhibitory antibodies directed against the heavy chain contribute for 45% to the inhibitory capacity of the patient's anti-factor VIII antibodies whereas anti-A3-C1-C2 antibodies account for the other half of the inhibitory capacity. Our results provide evidence for the occurrence of at least three classes of inhibitory antibodies in the patient's plasma. Next, we determined the subclass of the anti-factor VIII antibodies using methods that have been outlined previously (Fijnvandraat et al. 1997. Blood 89: 4371-4377). The antibodies with A2-specificity consisted predominantly of subclass IgG4; in addition small amounts of subclass IgG2 were observed. The antibodies directed against the factor VIII light chain consisted exclusively of subclass IgG4. The methods outlined above provide a starting point for further characterization of human antibodies with specificity for factor VIII. Similar analyses can be performed on samples derived of other patients which are analyzed for the presence of factor VIII inhibitors.

EXAMPLE 2: Construction of an IgG4 specific library

Peripheral blood lymphocytes were isolated from a blood sample of a patient with acquired haemophilia. The titre of the inhibitor was 1250 BU/ml. RNA was isolated from the lymphocytes using RNAzol (WAK Chemie, Germany) according to the instructions of the manufacturer. RNA was transcribed into cDNA employing random hexamer primers (Gibco, Breda, The Netherlands). Since, most of the anti-factor VIII antibodies described in Example 1 were of subclass IgG4, DNA fragments corresponding to the heavy chain of immunoglobulins of subclass IgG4 were amplified using the following set of oligonucleotide primers:

conIgG1-4	5'	CTTGTCCACCTTGGTGTTGCTGGG	3'
huIgG4	5'	ACGTTGCAGGTGTAGGTCTTC	3'
huVH1aback	5'	CAGGTGCAGCTGGTGCAGTCTGG	3'
huVH2aback	5'	CAGGTCAACTTAAGGGAGTCTGG	3'

huVH3aback	5'	GAGGTGCAGCTGGTGGAGTCTGG 3'
huVH4aback	5'	GAGGTGCAGCTGTTGCAGTCGGG 3'
huVH5aback	5'	GAGGTACAGCTGCAGCAGTCTGC 3'
huVH6aback	5'	CAGGTACAGCTGCAGCAGTCAGG 3'
huJH1-2forSal	5'	GAGTCATTCTCGTGTCGACACGGTGACCAGGGTGCC 3'
huJH3forSal	5'	GAGTCATTCTCGTGTCGACACGGTGACCATTGTCCC 3'
huJH4-5forSal	5'	GAGTCATTCTCGTGTCGACACGGTGACCAGGGTTCC 3'
huJH6forSal	5'	GAGTCATTCTCGTGTCGACACGGTGACCGTGGTCCC 3'
huVH1backNco	5'	AATCCATGGCCCAGGTGCAGCTGGTGCA 3'
huVH2backNco	5'	AATCCATGGCCCAGGTCAACTTAAGGGA 3'
huVH3backNco	5'	AATCCATGGCCGAGGTGCAGCTGGTGGA 3'
huVH4backNco	5'	AATCCATGGCCGAGGTGCAGCTGTTGCA 3'
huVH5backNco	5'	AATCCATGGCCGAGGTACAGCTGCAGCA 3'
huVH6backNco	5'	AATCCATGGCCCAGGTACAGCTGCAGCA 3'

Oligonucleotide primers huVHa(1-6)back and huJH(1-6)forSal have been described previously (Marks et al. 1991. J. Mol. Biol. 222: 581-597). Oligonucleotide primers huVH(1-6)backNco have been adapted from oligonucleotide primers described in the same paper. The first series of amplification involved primers huVH(1-6)back in conjunction with primer conIgG1-4. Six different DNA fragments of about 700 bp, each corresponding to an individual VH-gene family were obtained. The six different fragments were isolated and re-amplified with primers huVH(1-6)back and primer huIgG4. Six products of approximately 660 bp were obtained. The 6 different 660 bp fragments which represented the IgG4 repertoire of the patient were re-amplified with primers huVH(1-6)backNco and huJH(1-6)forSal in order to prepare these fragments for cloning. The resulting 24 fragments were pooled according to VH-gene family and the six different fragments were digested with NcoI and SalI. The digested fragments were purified and dissolved in TE (10 mM Tris-HCl pH=8.0; 0.1 mM EDTA). The vector pHEN-1-VLrep has been described previously (Griffin, H.M. and Ouwehand, W.H. 1995. Blood 86, 4430-4436; Schier et al. 1996. J. Mol. Biol. 255: 28-43) and contains a light chain repertoire derived of two non-immunized donors. Insertion of a heavy chain repertoire in this vector has been shown to result in the production of antibody fragments that consist of the variable domains of both heavy and light chain. These antibody fragments have been termed single chain Fv (scFv) fragments (Hoogenboom,

H.R. et al. 1991. Nucleic Acid Res. 19: 4133-4137). The vector pHEN-1-VLrep (kindly provided by Dr. W.H. Ouwehand, Department of Transfusion Medicine, University of Cambridge, UK) was digested with XhoI and NcoI and the six fragments corresponding to the IgG4-specific heavy chain repertoire of the patient with acquired haemophilia were inserted. The ligation mixtures were transformed to *E. coli* TG1 and a library of 1.500.000-2.500.000 independent clones was obtained. Colonies were scraped and resuspended in 2TY supplemented with 15% glycerol, 100 µg/ml ampicillin and 1% glucose. Similar to the methods outlined above libraries that represent the immunoglobulin repertoire of other patients may be assembled.

EXAMPLE 3: Selection of factor VIII specific antibodies

Selection of clones that encoded antibody fragments (scFvs) with factor VIII specificity was performed as outlined below. Glycerol stocks were plated onto 2TY plates that contained ampicillin (100 µg/ml) and 1% glucose. Colonies were grown overnight and scraped the next day and dissolved in 2TY supplemented with 100 µg/ml ampicillin and 1% glucose. These cells were diluted in 2TY supplemented with ampicillin (100 µg/ml) and 1% glucose till a final optical density (OD) of 0.3 (measured at 600 nm). Cells were grown at 37°C till an OD of 0.5. Subsequently, 1 ml of culture was diluted 10 times in 2TY with ampicillin (100 µg/ml) and 1% glucose. Next, 20 µl of helper phage was added (VCSM13; 1 x 10^{11} pfu/ml) and the mixture was incubated for 45 minutes at 37°C without shaking. Then, cells were incubated at 37°C with shaking at 150 rpm for another 45 minutes. The cells were spun down at low speed and resuspended in 100 ml of 2TY supplemented with ampicillin (100 µg/ml), 0.1% glucose and 25 µg/ml kanamycin. The cells were incubated overnight at 30°C. The next day cells were spun down at 10000 rpm for 30 minutes. The supernatant was harvested and recombinant phage were allowed to precipitate for 2 hours at 4°C after the addition of 1/5 volume of 20% PEG6000/2.5 M NaCl. The phages were spun down (30 minutes 10000 rpm) and resuspended in 5 ml of TBS (50 mM Tris-HCl pH 7.4, 150 mM NaCl). This preparation was spun down for 5 minutes at 14000 rpm and the supernatant was stored at 4°C.

Selection of factor VIII-binding phages was performed as outlined below. Microtiter wells were coated overnight at 4°C with 5 µg/ml of the murine factor VIII light chain specific monoclonal antibody CLB-CAg 12 which was diluted in 50 mM $NaHCO_3$ (pH 9.5). The wells were blocked for 1 hour at 37°C with TBS

containing 3% HSA. Phage solution (1×10^{12} pfu/ml) was diluted 1 to 1 in TBS supplemented with 6% HSA and 1% Tween-20 and incubated for 2 hours at room temperature with microtiter wells that contained immobilized CLB-CAg 12. The phage solution was removed and transferred to a second microtiter well (also coated with CLB-CAg 12) which had been preincubated with 1 µg/ml of factor VIII light chain. Phages were incubated with factor VIII light chain for 2 hours at room temperature. Wells were washed 20 times with TBS/0.1% Tween-20 and 20 times with TBS and bound phage was eluted with 100 mM triethylamine (pH 12). Eluted phage (volume 1 ml) was neutralized by the addition of 500 µl of 1 M Tris-HCl pH 7.4 and subsequently added to 5 ml of *E. coli* TG1 (OD 600 = 0.5). Cells were incubated for 1/2 hour at 37°C (no shaking) and 10 minutes at 37°C (shaken at 200 rpm). Cells were collected by centrifugation for 10 minutes at 4000 rpm for 7 minutes. Subsequently, infected TG1 cells were plated on 2TY agar plates supplemented with ampicillin (100 µg/ml) and 1.0% glucose. Cells were grown overnight at 30°C. Cells were scraped in 2TY supplemented with ampicillin (100 µg/ml), 1% glucose and 15% glycerol. Ampoules were stored at -70°C.

Alternatively, factor VIII light chain (5 µg/ml in 50 mM $NaHCO_3$ (pH 9.6)) was immobilized on immunotubes (Nunc, Life Technologies, Breda, The Netherlands). First, 1 ml of phage solution diluted in TBS supplemented with 3% HSA was incubated for 2 hours at room temperature in non-coated Immunotubes. Subsequently, 1 ml of phage solution was removed and incubated for 2 hours at room temperature in immunotubes coated with factor VIII light chain. Immunotubes were washed 20 times with TBS/0.1% Tween-20 and 20 times with TBS. Bound phage was eluted with 100 mM triethylamine (pH 12) and processed as outlined above.

The second round of panning was initiated by inoculating 50 µl of glycerol stock obtained after the first selection in 10 ml 2TY supplemented with ampicillin (100 µg/ml) and 1% glucose till a final OD600 of 0.3. Cells were grown till an OD600 of 0.5, diluted 1 to 10 in 2TY supplemented with ampicillin (100 µg/ml), kanamycin (25 µg/ml) and 1% glucose and subsequently infected with a 20 fold excess of VCSM13. Cells were grown overnight at 30°C. Supernatant containing the phage was harvested as described above and again screened for binding to immobilized factor VIII light chain. After four rounds of panning, 30 clones selected by ELISA and 30 clones that were obtained after panning with factor VIII light chain immobilized to immunotubes were grown

and analyzed for binding to the factor VIII light chain. Colonies were picked and grown overnight in 2 ml 2TY supplemented with 100 μg/ml ampicillin and 1% glucose. The next day the cultures were diluted 200 times and grown till an OD600 of approximately 0.5. Cells were subsequently infected with VCSM13 for 45 minutes at 37°C (no shaking) and 45 minutes at 37°C (shaken at 200 rpm). Infected cultures were diluted 1 to 10 in 2TY supplemented with 100 μg/ml ampicillin, 0.1% glucose and 25 μg/ml kanamycin and cells were grown overnight at 30°C. Supernatant containing phage was collected after centrifugation and tested for binding to the factor VIII light chain as described below. The non-inhibiting murine monoclonal antibody CLB-CAg 12 directed against an epitope in the A3-C1 domain of factor VIII was immobilized on microtiter wells at a concentration of 5 μg/ml in 50 mM $NaHCO_3$ (pH 9.5). Wells were blocked for 1 hour in TBS supplemented with 3% HSA. Wells were incubated with factor VIII light chain at a concentration of 1 μg/ml in 50 mM Tris HCl (pH 7.4), 1 M NaCl, 2% HSA for 2 hours at 37°C. Fifty μl of phage solution and an equal volume of TBS supplemented with 1% Tween-20 and 6% HSA were added to wells containing factor VIII light chain. To monitor specific binding of the phages, wells that did not contain factor VIII light chain were incubated simultaneously with the same phage solution. Phage was incubated at room temperature for 2 hours and were shaken at regular intervals. Subsequently, wells were extensively washed 5 times with TBS supplemented with 0.1% Tween-20 and washed 5 times with TBS. The presence of bound phage was monitored by incubating with a peroxidase labelled polyclonal antibody directed against M13 (Pharmacia-LKB, Woerden, The Netherlands) in a dilution of 1 to 4000 in TBS supplemented with 1% HSA and 0.1% Tween-20. Plates were washed 5 times with TBS supplemented with 0.1% Tween-20 and 5 times with TBS. Binding of peroxidase-labelled anti-M13 antibody was quantified by incubation with 3-3'-5-5' tetramethylbenzidine (TMB). Substrate conversion was arrested by the addition of 100 μl of 2N H_2SO_4. Part of the results of this analysis are given in Figure 1. An example of 12 clones that show specific binding to the factor VIII light chain is given. Clearly, phage encoded by clone 1 to 12 display binding to the factor VIII light chain (black bars). Some background binding is visible which is not dependent on the presence of the factor VIII light chain (grey bars). The bars labelled with c represent two clones that express antibody fragments that do not bind specifically to the factor VIII light chain. These clones have been derived from the initial library and have not been selected on the factor VIII light

chain. To ensure that during subsequent rounds of panning an increase in the amount of factor VIII specific recombinant antibody fragments was obtained, we screened 12 clones obtained after the first round of panning for binding to the factor VIII light chain (Figure 2). Only 3 out of 12 clones bind specifically to the factor VIII light chain. In 9 out of 12 clones binding of phage is not dependent on the presence of the factor VIII light chain. These results clearly indicate that during panning the amount of phages that express factor VIII-specific antibodies can be selectively enriched. In summary, we have outlined a specific protocol for the selection of factor VIII specific antibodies that correspond to the spectrum of anti-factor VIII antibodies present in the patient with acquired haemophilia. In the first two examples our analysis is limited to material derived of one single patient and only antibodies directed against the factor VIII light chain have been analyzed. Using the methods outlined in these two examples the repertoire of anti-factor VIII antibodies of other patients with an inhibitor can easily be obtained. Furthermore, anti-factor VIII antibodies directed against epitopes located outside the factor VIII light chain may be obtained by adapting the screening methods used in Example 1 and 2. Antibodies directed against the heavy chain can be selected by immobilizing factor VIII heavy chain employing monoclonal antibody CLB-CAg 9. In these two examples we have focused on the IgG4-repertoire of the patient. Similarly, other subclasses may be investigated using the appropriate primers. For example, subclass IgG1-4 can be detected by simply using primer conIgG1-4 described in Example 1. Similarly, other primers specific for IgA, IgM, IgE and IgD may be utilized to assemble antibody-repertoires that include factor VIII-specific antibodies.

EXAMPLE 4: Sequence characteristics of recombinant antibodies with factor VIII light chain specificity

In the previous examples methods to obtain recombinant antibodies with factor VIII specificity has been outlined. To obtain information on the properties of these antibodies we selected 30 clones that have been selected by immobilized factor VIII in immunotubes. Also 30 clones which were selected employing factor VIII light chain with monoclonal antibody CLB-CAg 12 were analyzed. Clones were grown as described in Example 2 and plasmid DNA was isolated. The nucleotide sequence of the variable part of the heavy chain (VH domain) of 55 clones was determined using fluorescently labelled M13 reverse primer on an ABI-Prism 377 DNA sequencer. The sequences obtained were aligned with

heavy chain sequences in the database "V BASE" of the MRC Centre of Protein Engineering (Cambridge, UK). The 55 clones analyzed were encoded by two different VH-gene segments DP-10 and DP-14 (Cook and Tomlinson, Immunology Today 16: 237-242). The 41 clones that were encoded by the germline sequence DP14 consisted of three groups of recombinant antibodies that differed mainly in the nucleotide sequences of the constant regions of the VH gene. Thirty-three clones which were represented by clone IT2, 5 clones were represented by EL25 and 3 clones were represented by clone EL5 (Table I). Two clones that were encoded by DP10 (EL14) and DP14 (IT2) were selected for further analysis. The nucleotide and primary amino acid sequence of these clones is listed in Figure 3 and 4. The characteristics of the two sequences are given in Table I. Part of clone EL14 is most likely derived of the D-segment D6-13 and J-segment JH-3b. Somatic hypermutation has occurred during the immune response as evidenced by the large number of nucleotide changes compared to the germline sequences of the VH segments. The variable heavy chain part of clone IT2 contains 20 nucleotide substitutions when compared to the germ line segment DP-14. These 20 nucleotide substitutions result in a total of 13 amino acid changes (Table I). The variable heavy chain part of clone EL14 contains 18 nucleotide substitutions when compared to the germ line segment DP-10. These 18 nucleotide substitutions result in 12 amino acid changes (Table I). Clone IT2 has in part been derived from gene segments D3-3 and JH6b. Remarkably, a stretch of G-residues is observed between the germ line sequences DP14 and D3-3 for clone IT2 that encodes for a flexible arm of glycine residues. Inspection of the amino acid sequence of clone EL14 and IT2 reveals several interesting features. Both CDR3 regions contain several glycine residues at their amino-terminal part which is in both cases followed by a tyrosine and a glutamic acid (GG-YE). Furthermore, a proline, alanine and an aspartic acid appear to be conserved in the carboxyl-terminal part of the CRD3 (P---A-D). A common motif can be derived from the amino acid sequences of the CDR3 regions of clone EL14 and IT2 which is given in Figure 4B. These features may determine the specificity of these antibodies for the factor VIII light chain. In this example the nucleotide and primary amino acid sequence of two recombinant factor VIII antibodies has been disclosed. With methods similar to the ones described in this example recombinant antibodies that are directed against other regions on the factor VIII molecule may be analyzed. Common features of these antibodies can be identified as outlined in this example and therapeutic and diagnostic agents

derived of these common features can be used for diagnosis and treatment of patients with factor VIII inhibitors.

EXAMPLE 5: Detection of nucleotide sequences of factor VIII specific antibodies in patient samples

The nucleotide and amino acid sequences outlines in the previous example can be used to specifically detect factor VIII antibodies with C2-specificity in heterogeneous mixtures of antibodies. This can be accomplished by developing reagents, for example, antibodies that specifically recognize the anti-factor VIII antibodies described in this invention. Detection of factor VIII-specific antibodies can also be performed by analysis of the presence of specific nucleotide sequences that encode factor VIII specific antibodies. Methods to obtain nucleotide sequences that encode factor VIII specific antibodies are disclosed in this invention. In this example the detection of nucleotide sequences encoding one of the factor VIII specific antibodies described in the previous example (EL14) is disclosed. Lymphocytes of the patient with acquired haemophilia described in the first example were obtained. RNA was isolated and cDNA was prepared. Subsequently, DNA fragments were amplified with oligonucleotide primers huVH(1-6)aback and conIgG1-4 (see Example 2). The six different 700 bp fragments obtained were isolated and used for a second PCR with oligonucleotide primer huVH(1-6)aback and huIgG4. This resulted in a fragment of 660 bp which was cloned into the vector pGEM-T (Promega, Madison, WI, USA). The presence of nucleotide sequences that corresponded to that of clone EL14 was addressed by nucleotide sequencing. One out of sixty clones analyzed did contain nucleotide sequences that were identical to that obtained for clone EL14. This analysis shows that, using the nucleotide sequences disclosed in this invention as a starting point, it is possible to monitor the presence of factor VIII specific antibodies in patient samples. In this example oligonucleotide primers are used which have also been employed for the construction of the IgG4 specific library. Other combinations of oligonucleotide primers that are based on the nucleotide sequences of clone EL14 and IT2 may be designed which may include but are not limited to oligonucleotide primers that are based upon the CDR3 region of these antibodies. In this example detection of factor VIII specific antibodies is performed using analysis of nucleotide sequences. Alternatively, detection of factor VIII specific sequences may also be performed employing selective hybridization using probes that are

based on the nucleotide sequence of the factor VIII specific antibodies disclosed in this invention. Other means of detection of specific nucleotide sequences that are known to an average expert in the art also fall within the scope of this invention. The methods disclosed in this invention allow for the isolation of factor VIII antibodies and determination of their nucleotide and amino acid sequence. In this example we have outlined described methods that detect factor VIII specific antibodies present in the repertoire of a patient with a factor VIII inhibitor. In examples 8 and 9 the nucleotide sequence of antibody fragments that bind to the A2- and A3-C1 domain of factor VIII is given. Methods similar to the ones described in this example can be used to detect nucleotide sequences that encode factor VIII inhibitors with A2-, A3-C1- or with a different epitope-specificity.

EXAMPLE 6: Properties of factor VIII-specific antibodies scFv-IT2 and scFv-EL14

The biochemical properties of the factor VIII specific antibodies IT2 and EL14 were characterized as follows. First, the plasmids pHEN-1-VL-EL14 and pHEN-1-VL-IT2 were digested with NcoI and NotI and the recombinant antibody fragments were isolated and cloned into the vector pUC119-sfi/Not-His6 (kindly provided by Dr. W.H. Ouwehand, University of Cambridge, Division of Transfusion Medicine, Cambridge UK). Positive clones were identified and grown till OD600 of 0.8-1.0 in 2TY medium supplemented with 1% glucose and 100 µg/ml ampicillin. Subsequently, Isopropyl-ß-D-thiogalactopyranoside (IPTG) till a final concentration of 1 mM was added and cells were grown for 3 hours at 30°C. Cells were harvested by centrifugation for 15 minutes at 4000 g at 4°C. The pellet was dissolved in 10 ml of 30 mM Tris-HCl (pH 8.0), 1 mM EDTA and 20% sucrose in order to release the content of the periplasma. The mixture was incubated at 4°C for 20 minutes and subsequently cells were collected by centrifugation (15 min 10800 g at 4°C). The supernatant which consists primarily of proteins present in periplasma was collected. The pellet was resuspended in 10 ml 5 mM $MgSO_4$ and incubated for 20 minutes at 4°C. Residual cell debris was collected by centrifugation for 15 minutes at 10800 g. The supernatant (designated osmotic shock fraction) was collected and added to the fraction containing periplasma-derived proteins. The pooled fractions were centrifuged for 20 minutes at 30000 g at 4°C and the supernatant was collected. The supernatant was filtered over a 0.22 µm filter. A

mixture of protease inhibitors was added (Complete™ Mini, Boehringer Mannheim, Germany) and the pooled fractions were dialysed overnight against a buffer containing 50 mM NaPi (pH 7.4), 20 mM imidazole and 500 mM NaCl. ScFv's were purified by nickel affinity resin Ni-NTA (QIAGEN, Germany) as follows: 1 ml of Ni-NTA matrix was equilibrated with 50 mM NaPi (pH 7.4), 250 mM imidazole, 500 mM NaCl and subsequently with 50 mM NaPi (pH 7.4), 20 mM imidazole, 500 mM NaCl. Dialysed supernatant containing factor VIII specific scFv's were then batch-wise incubated with Ni-NTA matrix for 3 hours at 4°C. The Ni-NTA was then transferred to a column and washed with 7 ml of 50 mM NaPi (pH 7.4), 20 mM imidazole, 500 mM NaCl and 7 ml of 50 mM NaPi (pH 7.4), 35 mM imidazole, 500 mM NaCl. ScFv's were eluted with 50 mM NaPi (pH 7.4), 250 mM imidazole, 500 mM NaCl and stored at 4°C. The purity of the different scFv preparations was addressed by SDS-PAGE followed by staining with Coomassie Brilliant Blue. All purified ScFv's appeared for at least 90% homogenous and migrated with an apparent molecular weight of 30 kDa. In the preparations obtained a small amount of a protein with a lower molecular weight was observed. The identity of this band was investigated by immunoblotting with monoclonal antibody 9E10. The epitope of this antibody is present at the carboxyl-terminus of the scFV's. Both the protein migrating at a molecular weight of 30 kDa and 15 kDa reacted with monoclonal antibody 9E10 on Western blot. This indicates that the 15 kDa fragment most likely corresponds to the light chain of the scFv's. Purified scFv's corresponding to clone IT2 and EL14 were purified as outlined above. A scFv derived of clone O4, a clone present in the patient library that did not bind to the factor VIII light chain was included as a negative control. The binding of scFv-IT2 and scFv-EL14 to the factor VIII light chain was addressed employing the following ELISA. Monoclonal antibody 9E10 (5 µg/ml) dissolved in 50 mM $NaHCO_3$ pH 9.5 was immobilized on microtiter wells overnight at 4°C. Subsequently, purified scFv's diluted in 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% HSA and 0.2% Tween-20 were added and incubated for 2 hours at room temperature. The microtiter plates were washed 5 times with TBS, 0.1% Tween-20. Next, purified factor VIII light chain was added (5 µg/ml) together with peroxidase labelled monoclonal antibody CLB-CAg A (0.5 µg/ml). The mixture (diluted in TBS, 0.1% Tween-20) was incubated for 2 hours at room temperature. The microtiter wells were washed 5 times with Tris-buffered saline (TBS) supplemented with 0.1% Tween-20 and 5 times with TBS. The amount of bound peroxidase labelled monoclonal

antibody CLB-CAg A was quantified by the substrate TMB. The results of this analysis are given in Figure 5. Both scFv-EL14 and scFv-IT2 react with specifically with the factor VIII light chain while scFv-04 did not react with the factor VIII light chain. Next, we used the factor VIII inhibitory murine monoclonal antibody CLB-CAg 117 for the detection of bound factor VIII light chain. The ELISA was performed as outlined above. Instead of peroxidase labelled CLB-CAg A we used peroxidase labelled CLB-CAg 117 for the detection of immobilized factor VIII light chain. We did not observe binding of CLB-CAg 117 when factor VIII light chain is immobilized by scFv-EL14 and scFv-IT2 (Figure 5). These results show that the epitope of scFv-EL14 and scFv-IT2 overlaps with that of CLB-CAg 117. Previously, we have shown that the inhibitory antibody CLB-CAg 117 is directed against the C2-domain of factor VIII (Fijnvandraat et al. 1998. Blood 91: 2347-2352). Apparently, both scFv's bind to an epitope in the C2-domain of factor VIII which overlaps with that of the inhibitory murine monoclonal antibody CLB-CAg 117.

Next, different dilutions of scFv-EL14 and scFv-IT2 were tested for binding to immobilized factor VIII light chain as outlined above using CLB-CAg A as the detecting antibody (Figure 6). From this analysis it appeared that scFv-EL14 binds with a higher affinity to the factor VIII light chain than scFv-IT2. These results were complemented by immunoprecipitation experiments for scFv-EL14. Immunoprecipitation experiments employing a metabolically labelled fragment corresponding to the C2-domain was performed essentially as described previously (Fijnvandraat et al. 1998. Blood 91: 2347-2352). Monoclonal antibody 9E10 was covalently linked to CNBr-activated Sepharose 4B and this matrix was used to bind scFv-EL14. Specific binding of scFv-EL14 to metabolically labelled C2-domain was detected and this confirms the C2-specificity of this recombinant antibody fragment. In this example methods have been disclosed to characterize recombinant antibodies with specificity for the C2-domain. In examples 8 and 9, we describe the nucleotide and amino acid sequence of recombinant antibody fragments that bind specifically to the A2- and A3-C1 domain of factor VIII. The methods described in this example can easily be adapted by an average expert skilled in the art, which will allow for characterization of recombinant antibodies directed against the A2, A3-C1 or another epitope on factor VIII.

EXAMPLE 7: Factor VIII specific recombinant antibody fragments scFv-IT2 and scFv-EL14 neutralize the activity of factor VIII inhibitors

In the previous example, we have shown that scFv-EL14 and scFv-IT2 bind to the factor VIII light chain and compete for binding with the murine inhibitory monoclonal antibody CLB-CAg 117. These observations suggest that the epitope of both scFv-EL14 and scFv-IT2 overlaps with that of CLB-CAg 117. It is expected that similar to CLB-CAg 117, scFv-EL14 and scFv-IT2 inhibit the biological activity of factor VIII. Increasing amounts of purified scFv's were tested for inhibition in the Bethesda assay. Surprisingly, addition of up to 170 μg/ml scFv did not result in factor VIII inhibition as measured in the Bethesda assay. In contrast, CLB-CAg 117 readily inhibited factor VIII when measured in the same assay. Apparently, binding of scFv-EL14 and scFv-IT2 to factor VIII does not interfere with the biological activity of factor VIII. This finding prompted us to investigate the capacity of both scFv-EL14 and scFv-IT2 to overcome inhibition by CLB-CAg 117. Monoclonal antibody CLB-CAg 117 was diluted till a final inhibitory activity of 2 BU/ml. This value corresponds with a residual factor VIII activity of 25% in the Bethesda assay. Subsequently, increasing concentrations of scFv-EL14 and scFv-IT2 were added. Surprisingly, both scFv-EL14 and scFv-IT2 could overcome the factor VIII inhibitory activity of CLB-CAg 117 (Figure 7). ScFv-EL14 (panel A) proved to be more efficient than scFv-IT2 (panel B) in neutralizing the inhibitory activity of CLB-CAg 117. Both scFv-EL14 and scFv-IT2 were unable to neutralize the inhibitory activity of monoclonal antibody CLB-CAg A, directed against amino acid residues Glu^{1811}-Lys^{1818} on the factor VIII light chain (Lenting et al. 1996. J. Biol. Chem. 271: 1935-1940). These results for the first time show that antibody fragments with factor VIII specificity can be used to interfere with the activity of factor VIII inhibitors. Administration of these antibody fragments will be beneficial for the treatment of patients with inhibitory antibodies directed against factor VIII. In this example the biological activity of antibody fragments with C2-specificity is disclosed. In examples 8 and 9, the nucleotide and amino acid sequence of recombinant antibody fragments that bind to the A2 and A3-C1 domain of factor VIII is disclosed. The methods disclosed in this and the previous example can easily be adapted by an average expert skilled in the art to establish the capacity of recombinant antibody fragments directed against the A2 or A3-C1 domain to neutralize factor VIII inhibitors. Similar to outlined in this example recombinant antibody fragments that bind to other regions can be evaluated for their

neutralizing capacity of factor VIII inhihitors. Similarly to what has been described in this example for scFv-EL14 and scFv-IT2, antibody fragments binding to A2, A3-C1 and other domains on factor VIII can be used for treatment of patients with factor VIII inhibitors.

EXAMPLE 8: Isolation and characteristics of anti-factor VIII antibodies that specifically bind to the A3-C1 domain of factor VIII.

Previous studies have indicated that plasma of a substantial number of inhibitor patients contains anti-factor VIII antibodies that bind specifically to the A3-C1 domains of factor VIII (Fijnvandraat et al. 1998. Blood 91: 2347-2352: Zhong et al. 1998. Blood 92: 136-142). Here, we have employed phage display technology to isolate anti-factor VIII antibodies from the total immunoglobulin repertoire of a haemophilia A patient with an inhibitor. Previously, we have shown that in plasma of this patient anti-factor VIII antibodies directed against the factor VIII light chain are present. The majority of the anti-factor VIII antibodies in this patient is directed against the A3-C1 domain whereas a small portion of anti-factor VIII antibodies reacts with the C2-domain (Fijnvandraat et al. 1998. Blood 91: 2347-2352). The majority of anti-factor VIII antibodies were of subclass IgG4. An IgG4-specific library was constructed using peripheral blood lymphocytes of the patient as starting material. A library consisting of 1.9 x 10^6 independent clones was obtained using the methods outlined in Example 2. Selection of recombinant phage that bind specifically to factor VIII was performed essentially as outlined in Example 3.

Microtiter wells were coated overnight at 4°C with 5 µg/ml of the murine factor VIII light chain specific monoclonal antibody CLB-CAg 117 which was diluted in 50 mM $NaHCO_3$ (pH 9.5). Monoclonal antibody CLB-CAg 117 is directed against the C2-domain of factor VIII. The use of CLB-CAg 117 in the selection-protocol may result in elimination of recombinant phages that express immunoglobulin fragments directed against the C2-domain of factor VIII. Recombinant phages expressing the IgG4 specific immunoglobulin repertoire were prepared as described in Example 3. Recombinant phages were initially incubated on microtiter wells that contained immobilized CLB-CAg 117 for 2 hours in TBS supplemented with 3% HSA and 0.5 % Tween-20. The phage solution was removed and transferred to a second microtiter well (also coated with CLB-CAg 117) which had been preincubated with 1 µg/ml of factor VIII

light chain. Phages were allowed to bind to the immobilized factor VIII light chain for 2 hours at room temperature. Wells were washed extensively as described in Example 3 and bound phage were eluted with 100 mM triethylamine (pH 12). The eluted phage were neutralized by the addition of 1 M Tris-HCl pH 7.4 and the resulting solution was used to infect E. coli TG1 cells as described in Example 3.

Alternatively, purified factor VIII (5 µg/ml in 50 mM $NaHCO_3$ (pH 9.6)) was immobilized on immunotubes (Nunc, Life Technologies, Breda, The Netherlands). Recombinant phages diluted in TBS supplemented with 3% HSA were first incubated for 2 hours at room temperature in non-coated immunotubes. Subsequently, 1 ml of phage solution was removed and incubated for 2 hours at room temperature in Immunotubes coated with factor VIII. Following extensive washing (20 times with TBS/0.1% Tween-20 and 20 times with TBS) bound phage were eluted with 100 mM triethylamine (pH 12) and processed as outlined above.

The second, third and fourth round of panning were performed using the selection protocol described above. After the fourth round of panning 15 individual clones were picked and recombinant phage were tested for binding to the factor VIII light chain and the A3-C1 domain. Factor VIII light chain was purified as described previously. A construct expressing recombinant A3-C1 domain was prepared essentially as described previously (Sawamoto et al. 1998. Thrombosis and Haemostasis vol. 78, 62-68) and expressed in CHO-cells. The non-inhibitory murine monoclonal antibody CLB-CAg 12 directed against an epitope in the A3-C1 domain of factor VIII was immobilized on microtiter wells at a concentration of 5 µg/ml in 50 mM $NaHCO_3$ (pH 9.5). Wells were blocked for 1 hour in TBS supplemented with 3% HSA. Subsequently, wells were incubated with factor VIII light chain (1 µg/ml) or recombinant A3-C1 domain (0.06 nM) in 50 mM Tris HCl (pH 7.4), 1 M NaCl, 2% HSA for 2 hours at 37°C. Fifty µl of phage solution and an equal volume of TBS supplemented with 1% Tween-20 and 6% HSA were added to wells containing immobilized factor VIII light chain or A3-C1 domain. To monitor specific binding of the phage, wells that did not contain factor VIII light chain or recombinant A3-C1 domain were incubated with simultaneously with the phage solution. Phage were incubated at room temperature for 2 hours and were shaken at regular intervals. Wells were washed extensively with TBS supplemented with 0.1 % Tween-20. The presence of bound phage was

monitored as described in Example 3. The results of the analysis are depicted in Figure 8. In panel A, 15 clones selected in immunotubes that contain factor VIII are depicted. Of the 15 clones analyzed, 1 clone (clone 20) did not react with factor VIII light chain and recombinant A3-C1 domain suggesting that this clone does not encode an antibody fragment with specificity for the A3-C1 domain of factor VIII. Three clones (clone 17, 23 and 24) do react with the factor VIII light chain but fail to react with recombinant A3-C1 domain. Apparently, the epitope of these recombinant antibody fragments is localized in the C2-domain of factor VIII. The remaining 11 clones react both with the factor VIII light chain and the recombinant A3-C1 domain. In panel B, 15 clones selected in microtiter wells that contain CLB-CAg 117 and factor VIII light chain are depicted. Clones 31-45 all interact with the factor VIII light chain (black bars) and recombinant A3-C1 domain (hatched bars). This analysis shows that phage derived of clone 31-45 encode antibody fragments that bind specifically to the A3-C1 domain of factor VIII.

These results show that the protocol outlined above is suitable for the selection of recombinant antibody fragments that bind specifically to the A3-C1 domain of factor VIII. Using the methods disclosed in this example, it is feasible to isolate recombinant phage encoding antibody fragments specific for the A3-C1 domain from other patients with factor VIII inhibitors.

The nucleotide sequence of the variable heavy chain fragments of 26 clones that reacted specifically with recombinant A3-C1 domain was determined essentially as described in Example 4. The sequences obtained were aligned with heavy chain sequences in the database "V BASE" of the MRC Centre of Protein Engineering (Cambridge, UK). The 26 clones analyzed were encoded by four different VH-gene segments DP15, DP31 and DP49 and DP77. The amino acid sequence of the variable heavy chain fragments of clones B38, B18, B35 and B04 is listed in Figure 9A. The nucleotide sequence of these four clones is presented in Figures 9B-E.

EXAMPLE 9: Isolation and characteristics of anti-factor VIII antibodies that bind to the A2-domain of factor VIII.

An immunodominant region which constitutes a binding site for factor VIII inhibitors has been localized to the A2-domain of factor VIII (Healey et al. 1995, J. Biol. Chem. 270: 14505-14509). We characterized the anti-factor VIII antibodies in plasma of a patient with mild haemophilia A and an inhibitor,

essentially as outlined in example 1. Recombinant factor VIII fragments corresponding to the A2, A3-C1-C2 and C2-domain of factor VIII were metabolically labelled with [^{35}S]-methionine and used for the detection of anti-factor VIII antibodies in the patient's plasma. Reactivity with metabolically labelled A2-domain and A3-C1-C2 domain was observed whereas only weak reactivity with metabolically labelled C2-domain was observed (data not shown). To determine the inhibitory capacity of both the anti-A2 and anti-A3-C1-C2 antibodies we performed neutralization experiments. The factor VIII inhibitor was diluted until a final value of 2 BU/ml and subsequently increasing amounts of recombinant A2 or A3-C1-C2 were added. Addition of recombinant A2-domain resulted in almost complete neutralization of the factor VIII inhibitors present in patient's plasma. Addition of recombinant A3-C1-C2 only neutralized the factor VIII inhibitor to a limited extent (< 10%). These results show that the majority of factor VIII inhibitors are directed towards the A2-domain of factor VIII. We assessed the subclass of the anti-factor VIII antibodies by enzyme linked sorbent assay. Both anti-A2 and anti-A3-C1-C2 antibodies consisted predominantly of subclass IgG4.

Peripheral blood lymphocytes of the patient were used to construct an IgG4-specific library as outlined in example 2. A library consisting of 1.9 x 10^6 clones was obtained. Recombinant phage expressing the IgG4-specific immunoglobulin repertoire of the patient were prepared as described in Example 3. Selection of phages binding to the A2-domain of factor VIII was performed by one of the following methods:

1. Purified factor VIII heavy chain (10 µg/ml) was immobilized on immunotubes (Nunc, Life Technologies, Breda, The Netherlands) in 50 mM $NaHCO_3$ (pH 9.5). Recombinant phages diluted in TBS supplemented with 3% HSA were first incubated for 2 hours at room temperature in non-coated immunotubes. Non-bound phage were transferred to an immunotube coated with factor VIII. Following extensive washing (20 times with TBS/0.1% Tween-20 and 20 times with TBS) bound phage was eluted with 100 mM triethylamine (pH 12). Eluted phage was neutralized by the addition of 1 M Tris-HCl pH 7.4 and used to infect E.coli TG1 cells as described in Example 3.

2. Alternatively, the murine monoclonal antibody CLB-CAg 9, directed against amino acid sequence 713-740 in the A2-domain of factor VIII was

immobilized on microtiter wells at a concentration of 5 µg/ml in 50 mM $NaHCO_3$ (pH 9.6). Purified factor VIII heavy chain (1 µg/ml) was then added and allowed to bind to CLB-CAg 9. Recombinant phage diluted in TBS 3% HSA and 0.5% Tween 20 were first incubated in microtiter wells containing only immobilized CLB-CAg 9. After 2 hours non-bound phage were transfered to a microtiter well which contained immobilized factor VIII heavy chain. Phage were allowed to bind to the factor VIII heavy chain for 2 hours at room temperature. Wells were washed extensively (see above) and finally bound phage were eluted with 100 mM triethylamine (pH 12) and processed as outlined above.

After four rounds of selection individual clones were picked and binding of recombinant phage to factor VIII heavy chain was evaluated by an enzyme linked immuno sorbent assay. Monoclonal antibody ESH5 (American Diagnostica, Greenwich, CT, USA) was immobilized onto microtiter wells at a concentration of 5 µg/ml in 50 mM $NaHCO_3$ (pH 9.6). Purified factor VIII heavy chain (1 µg/ml) was added and incubated for 2 hours at 37°C. Subsequently, recombinant phage, diluted 1 to 1 in 50 mM Tris-HCl pH 7.4, 1 M NaCl and 2% HSA, was added and incubated for 2 hours at room temperature. The amount of recombinant phage bound was determined was determined as described in Example 3. The results of the analysis is given in Figure 10. Twenty clones which were selected in microtiter wells in which factor VIII heavy chain was immobilized by CLB-CAg 9 were analyzed. Eleven out of 20 clones bound specifically to the heavy chain of factor VIII (Figure 10A). Clones that were selected by immobilized factor VIII heavy chain in immunotubes were also analyzed. Fifteen out of 20 clones bound specifically to the factor VIII heavy chain (Figure 10B). These results show that the protocol outlined above permits the isolation of anti-factor VIII antibodies that are directed against the heavy chain (A1-A2) of factor VIII. Using the methods disclosed in this example it is feasible to isolate anti-factor VIII antibodies from the repertoire of additional patients with factor VIII inhibitors directed against the A2-domain.

The nucleotide sequence of the variable heavy chain fragments of 26 clones that reacted specifically with the factor VIII heavy chain were determined essentially as described in Example 4. The sequences obtained were aligned with heavy chain sequences in the database "V BASE"of the MRC

Centre of Protein Engineering (Cambridge, UK). The 26 clones analyzed were encoded by two different VH-gene segments DP10 and DP47 (Figure 11A). The nucleotide sequence of the variable heavy chain of these clones is listed in Figure 11B and C.

EXAMPLE 10: Factor VIII specific recombinant antibody fragments can neutralize the activity of factor VIII inhibitors present in plasma of patients with haemophilia.

In example 7, we have shown that scFv-EL14 and scFv-IT2 neutralize the inhibitory activity of the murine monoclonal antibody CLB-CAg 117. We tested whether scFv-EL14 can also neutralize factor VIII inhibitors present in plasma of haemophilia A patients. First, we tested plasma of the patient with acquired haemophilia from whom the recombinant antibody fragments were derived. As described in example 1, recombinant C2-domain was capable of neutralizing 20% of the factor VIII inhibitor in patient's plasma (Table III). The effect of scFv-EL14 was evaluated in a similar set-up. Plasma was diluted till a final value of 2 BU/ml and increasing amounts of scFv-EL14 were added. ScFv-EL14 could neutralize about 20% of the total activity of factor VIII inhibitor in patient's plasma. These results suggest that scFv directed against the C2-domain prevent binding of factor VIII inhibitory antibodies that bind to the C2-domain of factor VIII.

Next, we tested two plasma samples derived of patients with congenital haemophilia A and factor VIII inhibitors. The relative contribution of the C2-domain to the total amount of factor VIII inhibitor for both samples ranged between 40% and 90%. Neutralization experiments indicate that addition of increasing concentrations of scFv-EL14 results in significant reduction of the levels of factor VIII inhibitor in plasma of these two patients with congenital haemophilia A. These results confirm that scFv-EL14 alleviates binding of human factor VIII inhibitors to the C2-domain. Our findings show that scFv-EL14 shields antigenic sites that are present in the C2-domain of factor VIII. This property of scFv-EL14 can be utilized to prevent binding of factor VIII inhibitors to the C2-domain of administered factor VIII in haemophilia A patients with an inhibitor. It has been firmly established that factor VIII inhibitors often recognize multiple epitopes that have been localized to the A2-, A3- and C2 domain of factor VIII. In this example the neutralizing activity of the C2-domain specific scFv-EL14 on the biological activity of factor VIII

inhibitors is described.

In examples 8 and 9 we have disclosed methods to obtain recombinant antibodies that specifically react with the A3-C1 domain and heavy chain (A1-A2) of factor VIII. In this example, we have shown that recombinant antibodies directed against the C2-domain of factor VIII can shield antigenic sites on factor VIII. Similarly, anti-A3-C1 antibodies and anti-A2-antibodies described in examples 8 and 9 can be tested for their ability to compete with factor VIII inhibitors for binding to factor VIII. The anti-A3-C1 and anti-A1-A2 antibodies disclosed in this invention may also be used for treatment of patients with inhibitors, which react with the A3-C1 and/or the A1-A2-domain.

Table I: Nucleotide sequences of clones expressing recombinant antibodies with specificity for the factor VIII light chain. Based on the nucleotide sequence 55 of the 60 clones analyzed could be arranged as depicted below. In the first column clones with the same nucleotide sequence are arranged in four groups. The number of clones corresponding to this group is given in brackets. Clone EL5, EL25 and IT2 are related as indicated in Figure 4B. In the second column the heavy chain family to which these clones belong is depicted. All clones analyzed belong to the VH1-family. In the third column the germline segment is depicted. Clone EL5, El25 and IT2 belong to germline segment DP-14 while clone EL14 belongs to germline sequence DP-10. In the fourth column the number of mutations in the different clones is depicted. The first number corresponds to the number of nucleotide mutations while the second one corresponds to the number of amino acid changes. The sequences were compared with the nucleotide and amino acid sequences of the germline segments indicated in the Table.

Clone	VH family	Germline segment	Mutations
EL5 (3)	VH1	DP-14	20/12
EL14 (14)	VH1	DP-10	18/12
EL25 (5)	VH1	DP-14	19/11
IT2 (33)	VH1	DP-14	20/13

CLAIMS

1. A polynucleotide in substantially isolated form, comprising a contiguous nucleotide sequence (a) coding for a human antibody with factor VIII specificity, or (b) complementary to a nucleotide sequence coding for a human antibody with factor VIII specificity, or (c) capable of selectively hybridizing under stringent conditions to nucleotide sequence (a) or (b).

2. A polynucleotide according to claim 1, wherein said contiguous nucleotide sequence is at least 8, preferably at least 10 nucleotides.

3. A probe or primer which comprises a polynucleotide according to claim 1 or claim 2, optionally further comprising a detectable label, such as a radioactive atom or group, an enzyme, a fluorescent or luminescent group, a dye or biotin.

4. An assay kit for detecting nucleic acid coding for a human antibody with factor VIII specificity, comprising a probe or primer according to claim 3 in a suitable container.

5. A nucleic acid amplification and detection kit for detecting nucleic acid coding for a human antibody with factor VIII specificity, comprising a pair of primers according to claim 3 capable of priming the synthesis of cDNA, and optionally further comprising a probe according to claim 3 capable of selectively hybridizing to (the complement of) a region of the nucleic acid to be detected between and not including the sequences from which the primers are derived.

6. A method for assaying a sample for the presence or absence of nucleic acid coding for a human antibody with factor VIII specificity, comprising contacting the sample with a probe according to claim 3 under conditions that allow the selective hybridization of said probe to the (complement of the) nucleic acid to be detected in the sample, and determining whether polynucleotide duplexes comprising said probe are formed.

7. A method for assaying a sample for the presence or absence of nucleic acid coding for a human antibody with factor VIII specificity, comprising subjecting nucleic acid present in the sample to a nucleic acid amplification process using a pair of primers according to claim 3 capable of priming the synthesis of cDNA, contacting the nucleic acid resulting from the amplification process with a probe according to claim 3 under conditions that allow the selective hybridization of said probe to the (complement of the) nucleic acid to

be detected in the sample, and determining whether polynucleotide duplexes comprising said probe are formed.

8. A method of producing a recombinant polypeptide, comprising providing a polynucleotide coding for said polypeptide, preparing a recombinant vector containing said polynucleotide operably linked to a control sequence capable of providing for the expression of the polynucleotide by a host cell, transforming a host cell with said recombinant vector, growing said host cell under conditions that provide for the expression of the polynucleotide and optionally isolating the thus produced polypeptide, wherein said polynucleotide codes for a human antibody with factor VIII specificity, or a fragment or derivative thereof capable of specific binding to factor VIII.

9. A polypeptide in substantially isolated form, comprising a contiguous amino acid sequence corresponding to or mimicking a fragment or derivative of a human antibody with factor VIII specificity capable of specific binding to factor VIII.

10. A polypeptide according to claim 9, wherein said contiguous amino acid sequence is capable of reducing the activity of factor VIII inhibiting antibodies.

11. A polypeptide according to claim 9 or claim 10, wherein said fragment is (part of) a variable region of the heavy chain or light chain of said antibody.

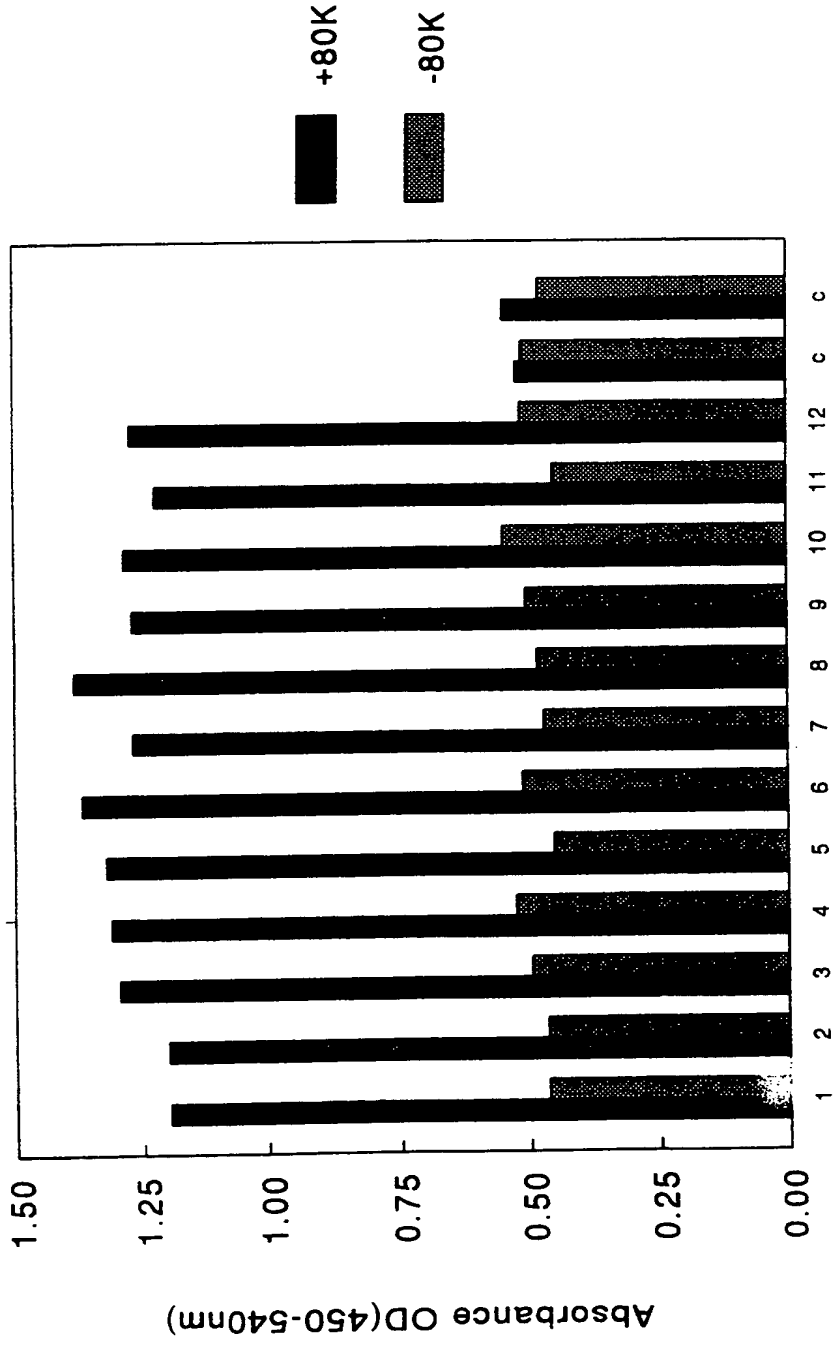
12. A polypeptide according to claim 9 or claim 10, wherein said derivative is a single chain Fv fragment of said antibody.

13. An antibody in substantially isolated form, comprising a recombinant human antibody with factor VIII specificity or an anti-idiotypic antibody directed against a human antibody with factor VIII specificity.

14. A pharmaceutical composition for the treatment of factor VIII inhibition in a human individual, comprising a polypeptide according to any one of claims 9-12 or an antibody according to claim 13, together with a pharmaceutically acceptable carrier.

15. A composition according to claim 14, which further contains factor VIII or a substitute of factor VIII.

16. A method of treatment of factor VIII inhibition in a human individual, comprising administering to said individual a polypeptide according to any one of claims 9-12 or an antibody according to claim 13, optionally together with factor VIII or a substitute of factor VIII.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1.25
1.00
0.75
0.50
0.25
0.00
Absorbance OD(450-540nm)
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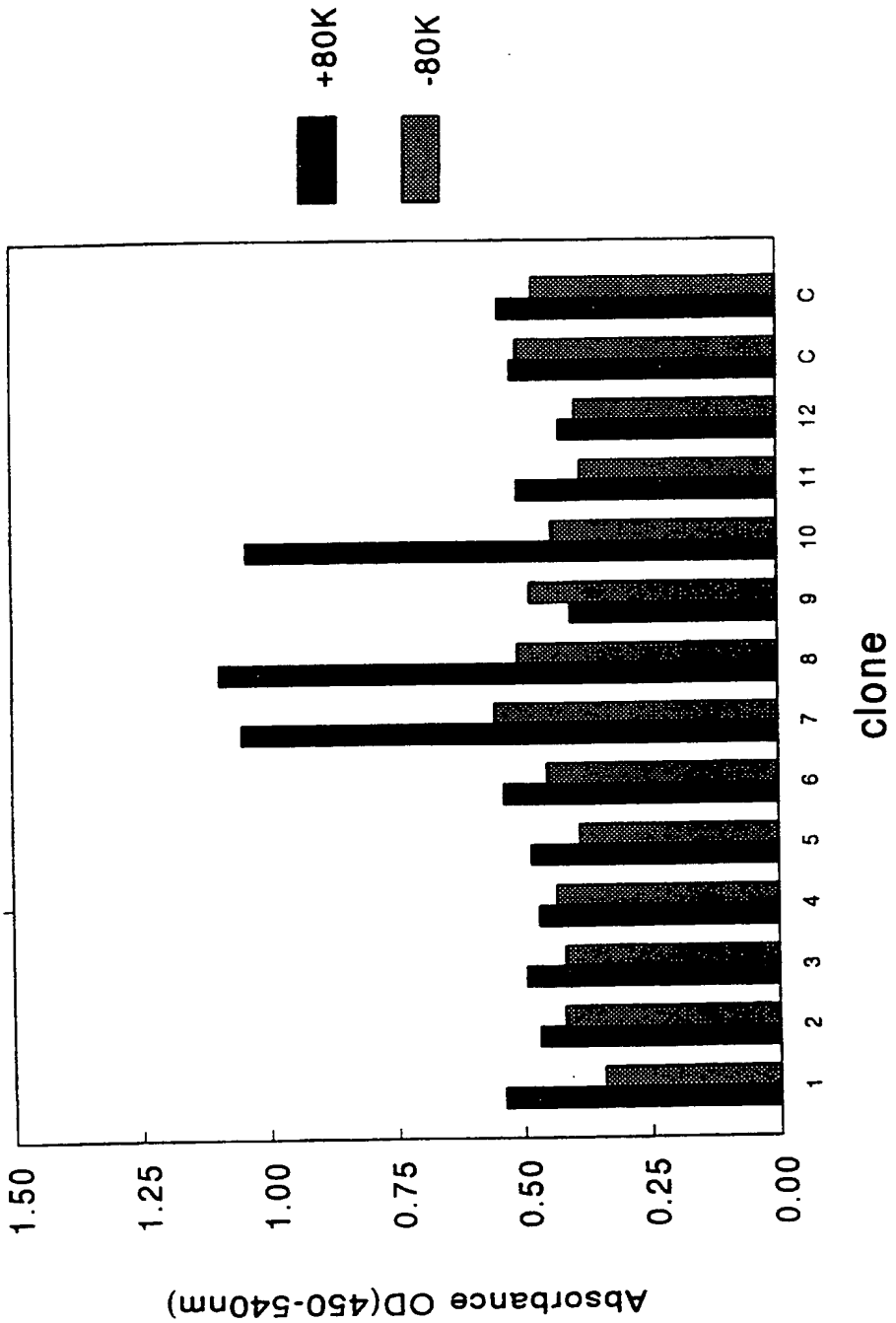
9
10
11
12
c
c
c
clone

Figure 1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-80K
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5
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7
8
9
10
11
12
C
C
clone

Figure 2

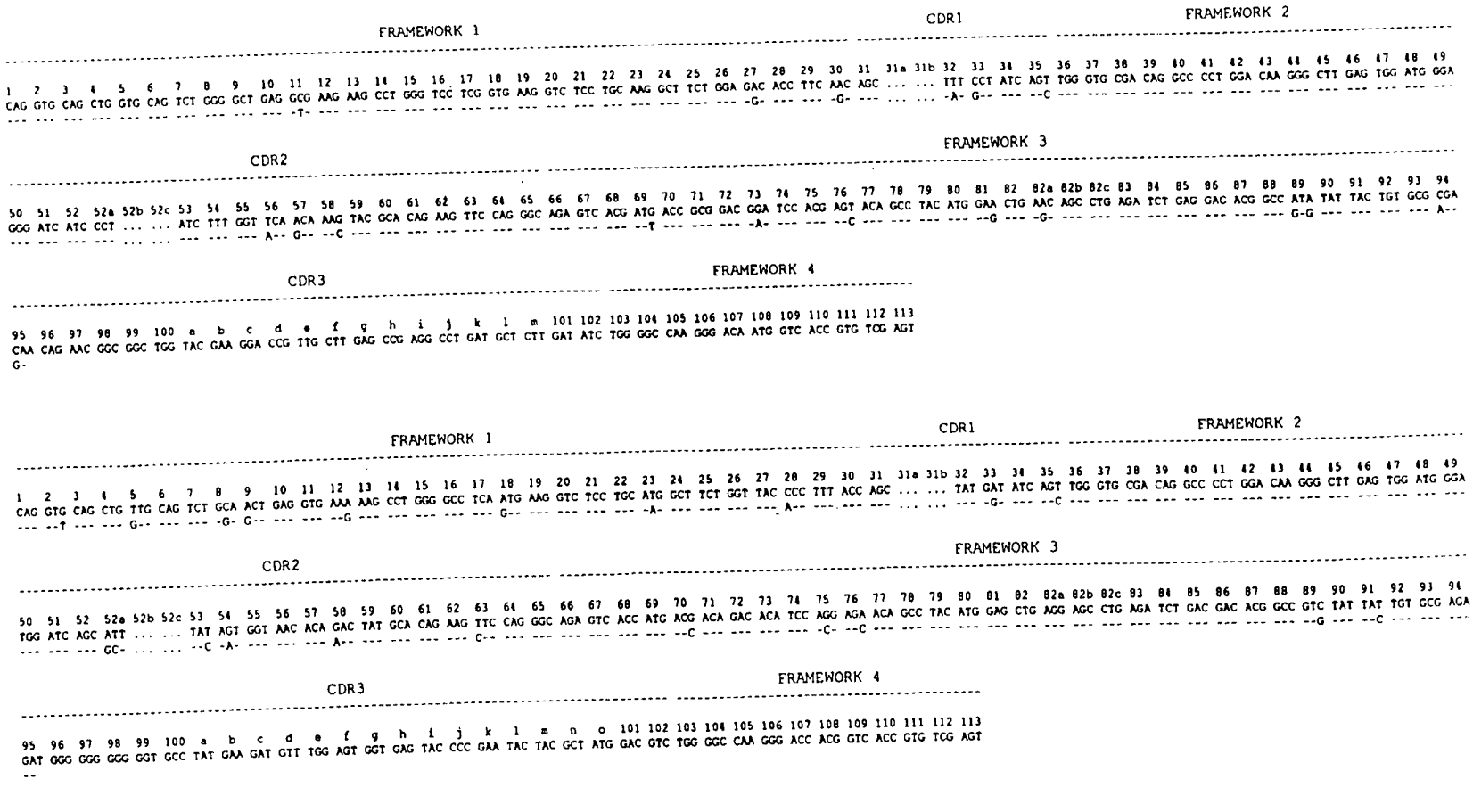


Figure 3

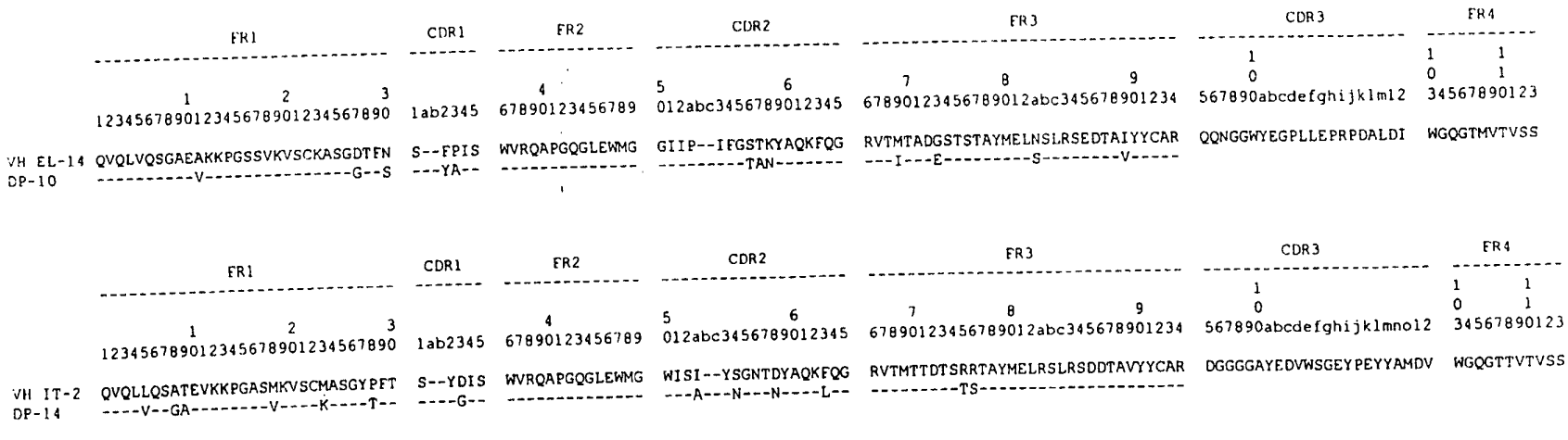


Figure 4A

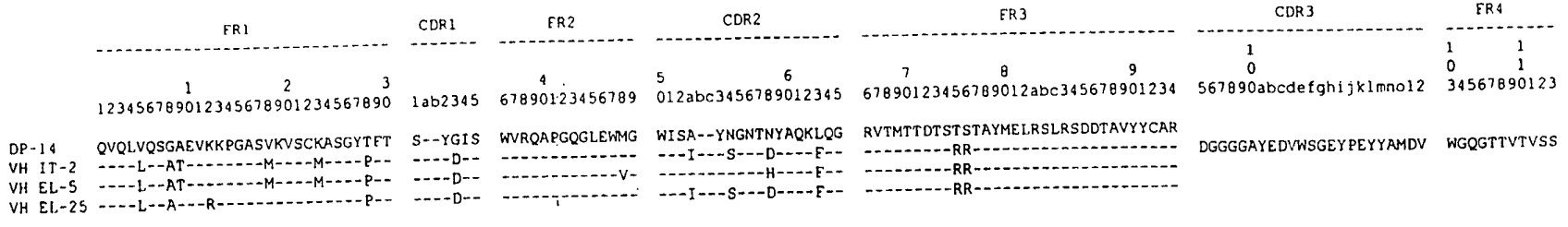
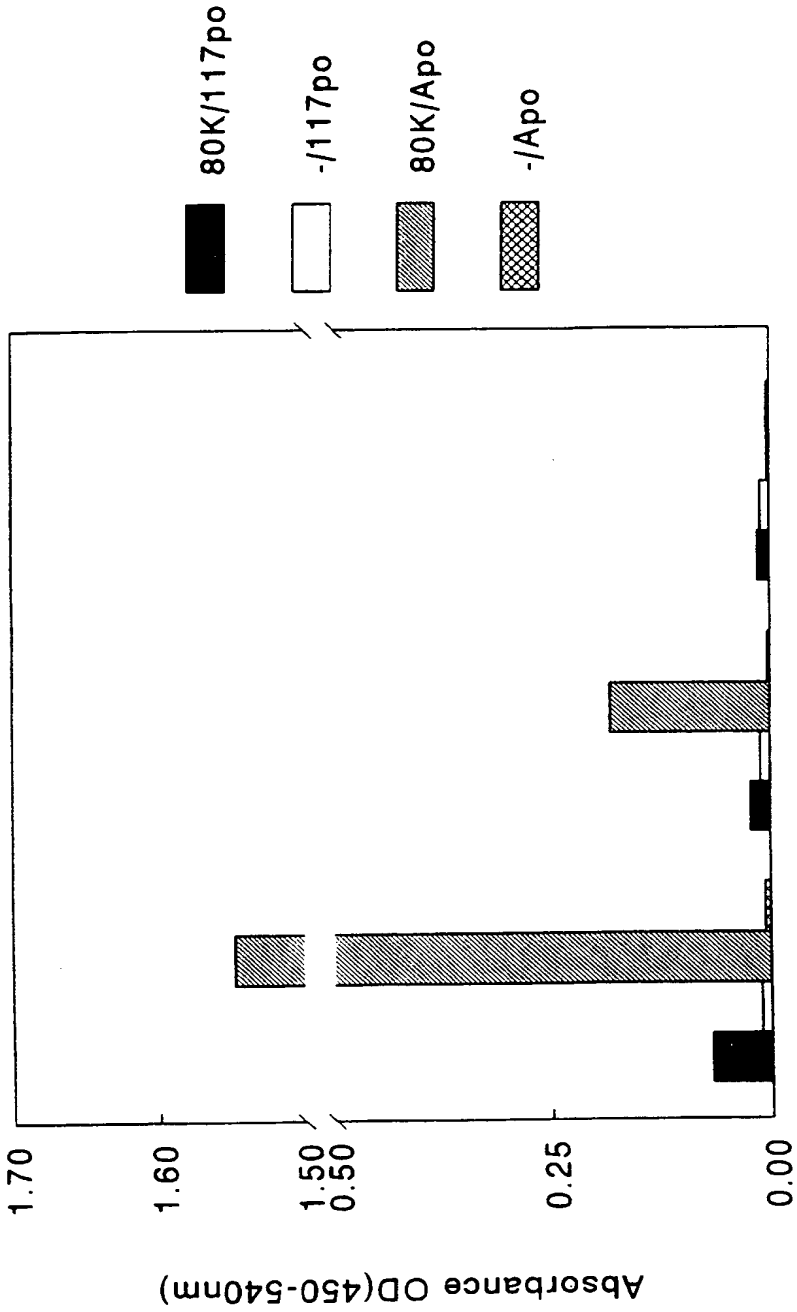

FR1
CDR1
FR2
CDR2
FR3
CDR3
FR4
1 2 3
4
5 6
7 8 9
1
0
1 1
0 1
12345678901234567890123456789
1ab2345
678901234567 89
012abc3456789012345
678901234567890123abc345678901234
567890abcdefghijklmnol2
34567890123
DP-14 QVQLVQSGAEVKKPGASVKVSCKASGYTFT S--YGIS WVRQAPGQGLEWMG WISA--YNGNTNYAQKLQG RVTMTTDTSTSTAYMELRSLRSDDTAVYYCAR DGGGGAYEDVWSGEYPEYYAMDV WGQGTTVTVSS
VH IT-2 ----L--AT--------M----M----P-- ----D-- ---------- ---I---S---D----F-- ---------RR----------
VH EL-5 ----L--AT--------M----M----P-- ----D-- ----------V- ----------H----F-- ---------RR----------
VH EL-25 ----L--A---R----------P-- ----D-- ---------- ---I---S---D----F-- ---------RR----------

Figure 4B

```
                CDR3

EL-14   QQNGGWYEGPLLEPRPD--ALDI
           || ||       |.  |.|.
IT-2    DGGGGAYEDVWSGEYPEYYAMDV
```

Figure 4C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-/117po
80K/Apo
-/Apo
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1.50
0.50
0.25
0.00
EL14
IT2
04
scFvs

Figure 5

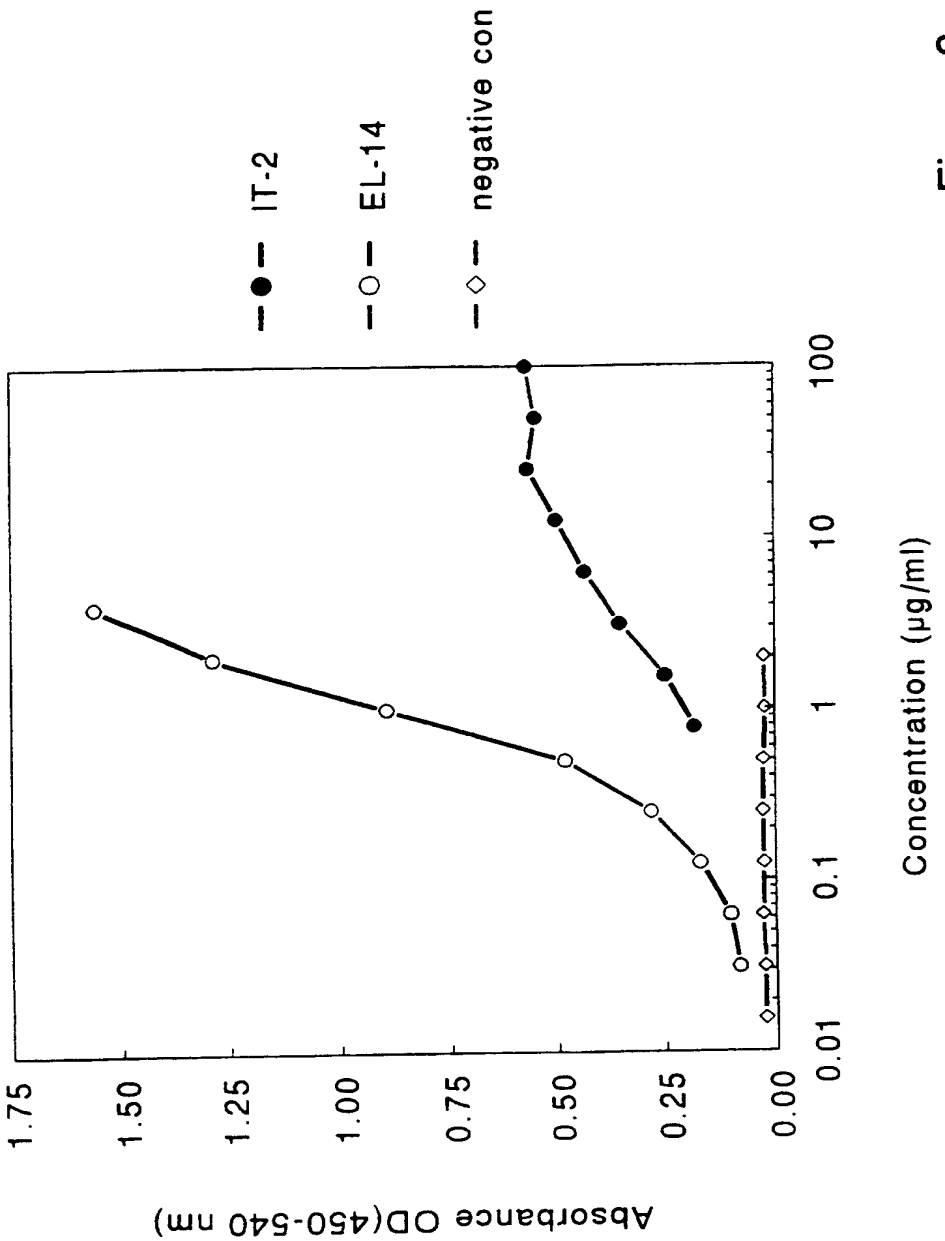

IT-2
EL-14
negative con
Absorbance OD(450-540 nm)
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
0.01
0.1
1
10
100
Concentration (µg/ml)

Figure 6

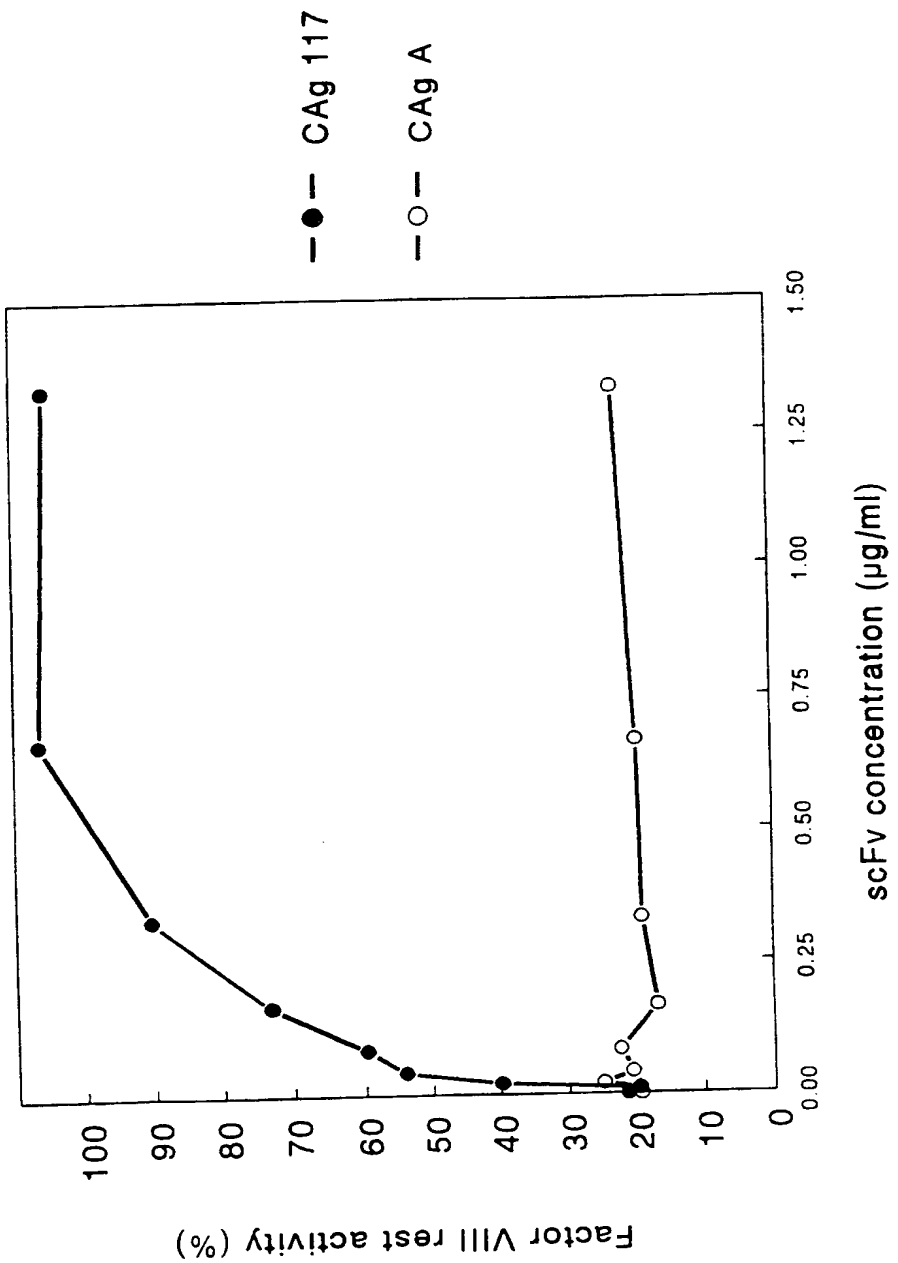


Figure 7A NEUTRALIZATION BY scFv EL-14

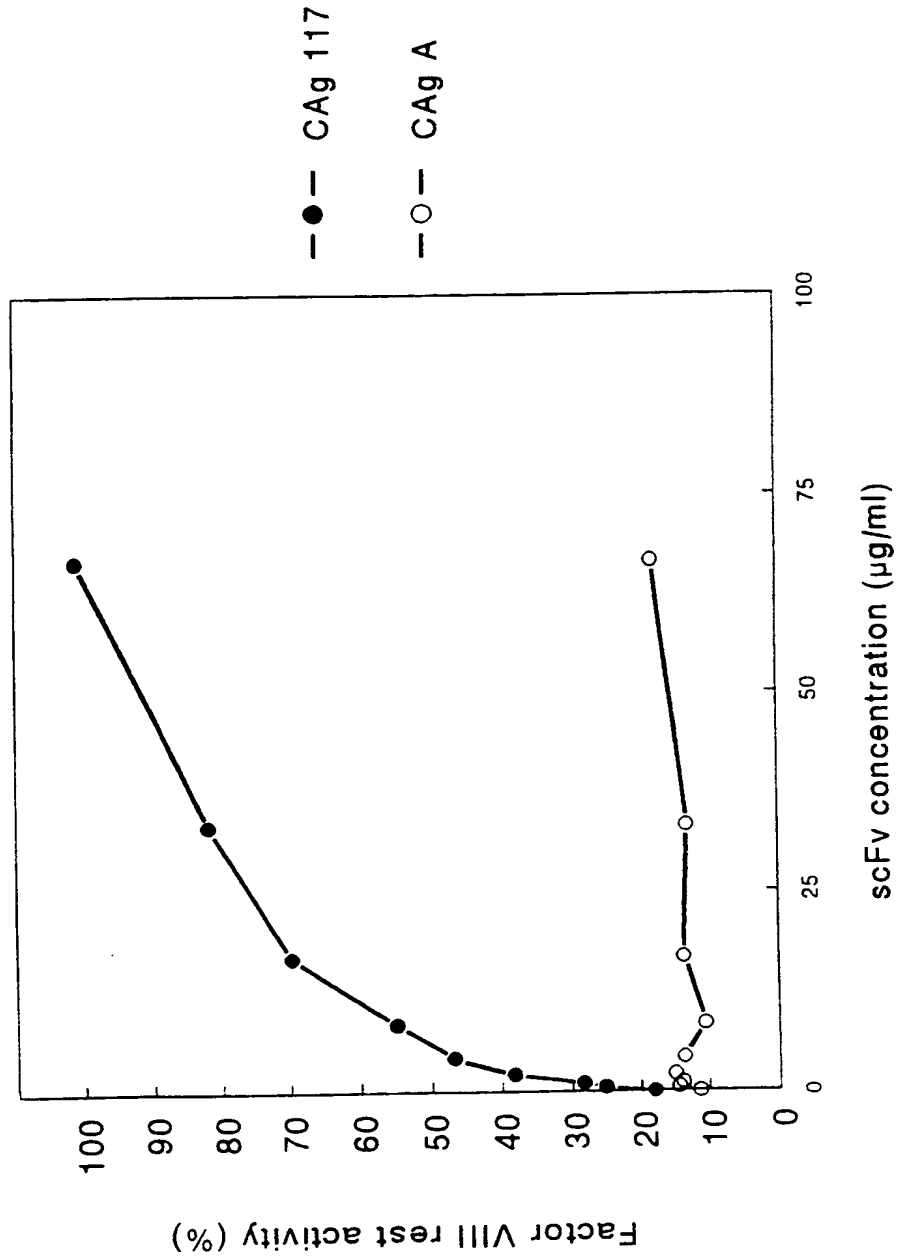
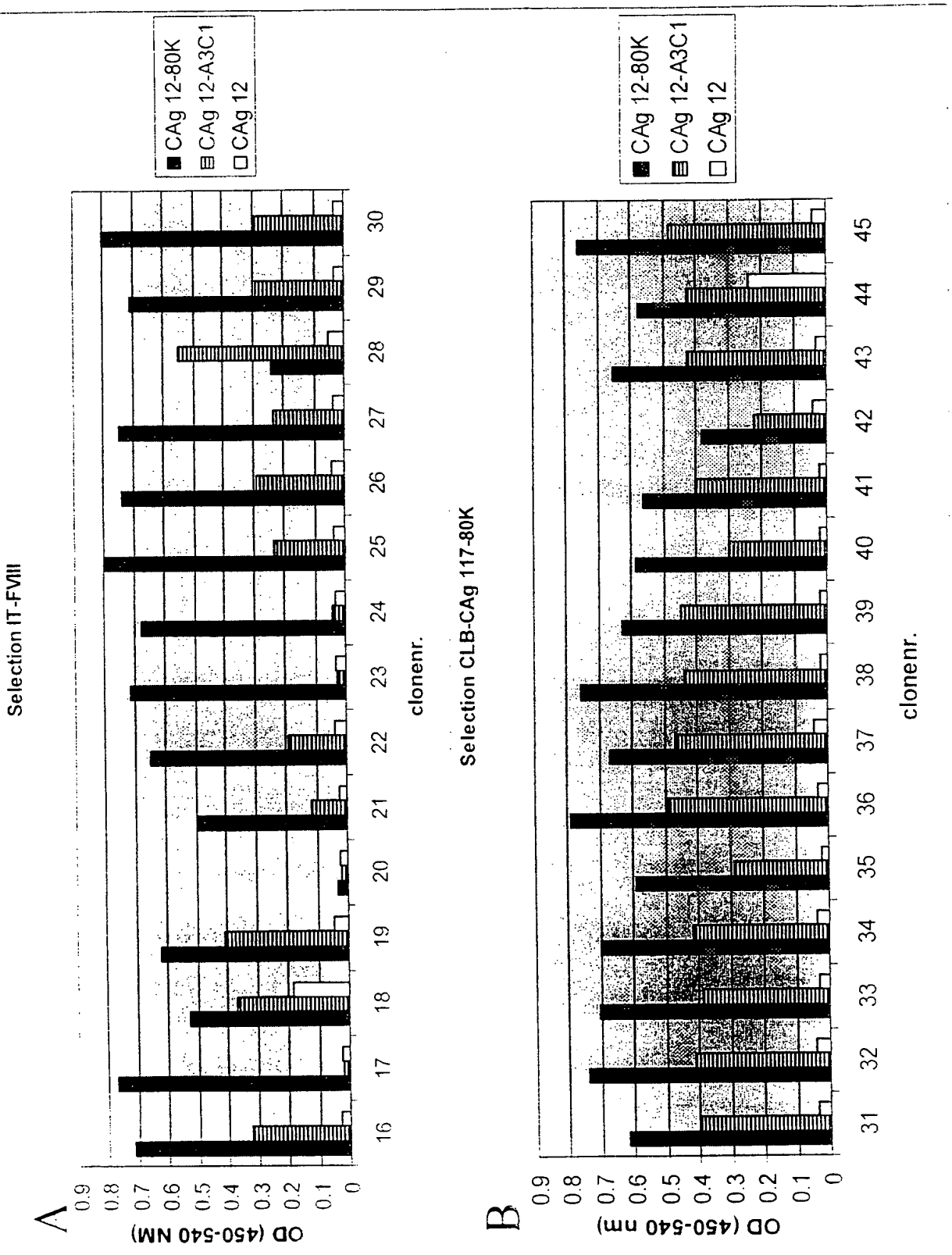


Figure 7B NEUTRALIZATION BY scFv IT-2

Figure 8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Selection IT-FVIII
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CAg 12-A3C1
CAg 12
OD (450-540 NM)
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0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
clonenr.
B
Selection CLB-CAg 117-80K
CAg 12-80K
CAg 12-A3C1
CAg 12
OD (450-540 nm)
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
clonenr.

Deduced protein sequences of isolated FVIII A3-C1 specific scFv

Heavy chains

```
                  FR1                        CDR1      FR2            CDR2                          FR3                     CDR3          FR4
                                                                                                                            1              1      1
               1           2           3               4          5           6             7          8            9       0              0      1
      1234567890123456789012345678901234567890 12345 67890123456789 012a3456789012345 678901234567890l2abc345678901234 567890abcdefghi12 34567890123

DP-15 QVQLVQSGAEVKKPGASVKVSCKASGYTFT SYDIN WVRQATGQGLEWMG WMNPNSGNTGYAQKFQG RVTMTRNTSISTAYMELSSLRSEDTAVYYCAR
B38   ----L-YA-D-----------T----I-- ----- -------------- --------A-F----K- -L-L--D--T-------RN-E---------- CDTTLLIWFGPAPYNDS WGQGTLV

DP-31 EVQLVESGGGLVQPGRSLRLSCAASGFTFD DYAMH WVRQAPGKGLEWVS GISWNSGSIGYADSVKG RFTISRDNAKNSLYLQMNSLRAEDTALYYCAK D
B18   Q----Q---------K------------G ---I- -------E------ -VT-SGTT--F------ ---------------Y---------------L PYINSSNYRRGVAAFDI WGQGTMVTVSS

DP-49 QVQLVESGGGVVQPGRSLRLSCAASGFTFS SYGMH WVRQAPGKGLEWVA VISYDGSNKYYADSVKG RFTISRDNSKNTLYLQMNSLRAEDTAVYYCAK
B35   E---------L-----------VD--L--- ----- -------A------ ------ND--------- --A-----A-----------TI---------- DLIESNI      AEAL WGQGTLVTVSS

DP-77 EVQLVESGGGLVKPGGSLRLSCAASGFTFS SYSMN WVRQAPGKGLEWVS SISSSSSYIYYADSVKG RFTISRDNAKNSLYLQMNSLRAEDTAVYYCAR
B04   -----K--E-------------------R R-DIH ----T--------- ----GGN--D------- ---------N-VV-----------M---F--- DGTIFGSAATWR AFDI WGRGTMVTVSSG
```

Figure 9A

```
+1   Gln Val Gln Leu Leu Gln Ser Ala Ala Asp Val Lys Lys Pro Gly Ala Ser
     CAGGTGCAGC TGTTGCAGTC TGCAGCTGAC GTGAAGAAGC CTGGGGCCTC 50
     GTCCACGTCG ACAACGTCAG ACGTCGACTG CACTTCTTCG GACCCCGGAG

+1   - Val Lys Val Ser Cys Thr Ala Ser Gly Tyr Ile Phe Thr Ser Tyr Asp Ile
     AGTGAAGGTC TCCTGTACGG CTTCTGGATA CATCTTCACC AGTTATGATA 100
     TCACTTCCAG AGGACATGCC GAAGACCTAT GTAGAAGTGG TCAATACTAT

+1   -- Asn Trp Val Arg Gln Ala Thr Gly Gln Gly Leu Glu Trp Met Gly Trp
     TCAACTGGGT GCGACAGGCC ACTGGACAAG GGCTTGAGTG GATGGGATGG 150
     AGTTGACCCA CGCTGTCCGG TGACCTGTTC CCGAACTCAC CTACCCTACC

+1   Met Asn Pro Asn Ser Gly Asn Ala Gly Phe Ala Gln Lys Phe Lys Gly Arg
     ATGAATCCTA ACAGTGGTAA CGCAGGCTTT GCACAGAAGT TTAAGGGCAG 200
     TACTTAGGAT TGTCACCATT GCGTCCGAAA CGTGTCTTCA AATTCCCGTC

+1   - Leu Thr Leu Thr Arg Asp Thr Ser Thr Ser Thr Ala Tyr Met Glu Leu Arg
     ACTCACCTTG ACCAGGGACA CTTCCACAAG CACAGCCTAC ATGGAGCTGA 250
     TGAGTGGAAC TGGTCCCTGT GAAGGTGTTC GTGTCGGATG TACCTCGACT

+1   -- Arg Leu Glu Ser Glu Asp Thr Ala Val Tyr Tyr Cys Ala Arg Cys Asp
     GGAGACTGGA ATCTGAGGAC ACGGCCGTGT ATTACTGTGC GAGATGTGAC 300
     CCTCTGACCT TAGACTCCTG TGCCGGCACA TAATGACACG CTCTACACTG

+1   Thr Thr Leu Leu Ile Trp Phe Gly Pro Ala Pro Tyr Tyr Asp Ser Trp Gly
     ACCACACTCT TAATCTGGTT CGGGCCCGCC CCCTACTATG ACTCCTGGGG 350
     TGGTGTGAGA ATTAGACCAA GCCCGGGCGG GGGATGATAC TGAGGACCCC

+1   - Gln Gly Thr Leu Val
     CCAGGGAACT CTAGTC                                      400
     GGTCCCTTGA GATCAG
```

Figure 9B

```
+1  Gln Val Gln Leu Val Gln Ser Gly Gly Gly  Leu Val Gln Pro Gly Lys Ser
    CAGGTGCAAC TGGTGCAGTC TGGGGGAGGC TTGGTACAGC CTGGCAAGTC 50
    GTCCACGTTG ACCACGTCAG ACCCCCTCCG AACCATGTCG GACCGTTCAG

+1  Leu Arg Leu  Ser Cys Ala Ala Ser Gly Phe Thr Phe Gly  Asp Tyr Ala Ile
    CCTGAGACTC TCCTGTGCAG CCTCTGGATT CACATTTGGC GATTATGCCA 100
    GGACTCTGAG AGGACACGTC GGAGACCTAA GTGTAAACCG CTAATACGGT

+1  His Trp Val Arg Gln Ala  Pro Gly Glu Gly Leu Glu Trp Val Ser Gly
    TACACTGGGT CCGGCAAGCT CCAGGGGAGG GCCTGGAGTG GGTCTCAGGT 150
    ATGTGACCCA GGCCGTTCGA GGTCCCCTCC CGGACCTCAC CCAGAGTCCA

+1  Val Thr Trp Ser Gly Thr Thr Ile Gly Phe  Ala Asp Ser Val Lys Gly Arg
    GTTACTTGGA GTGGTACTAC TATAGGCTTT GCGGACTCTG TGAAGGGCCG 200
    CAATGAACCT CACCATGATG ATATCCGAAA CGCCTGAGAC ACTTCCCGGC

+1  Phe Thr Ile  Ser Arg Asp Asn Ala Lys Asn Ser Leu Tyr  Leu Tyr Met Asn
    ATTCACCATC TCCAGAGACA ACGCCAAGAA TTCCCTGTAT CTGTACATGA 250
    TAAGTGGTAG AGGTCTCTGT TGCGGTTCTT AAGGGACATA GACATGTACT

+1  Ser Leu Arg Ala Glu Asp  Thr Ala Leu Tyr Tyr Cys Ala Leu Pro Tyr
    ACAGTCTGAG AGCTGAAGAC ACGGCCTTGT ATTATTGTGC CTTACCATAT 300
    TGTCAGACTC TCGACTTCTG TGCCGGAACA TAATAACACG GAATGGTATA

+1  Ile Asn Ser Ser Asn Tyr Arg Arg Gly Val  Ala Ala Phe Asp Ile Trp Gly
    ATCAACTCGT CCAACTACAG AAGAGGGGTC GCTGCCTTCG ATATCTGGGG 350
    TAGTTGAGCA GGTTGATGTC TTCTCCCCAG CGACGGAAGC TATAGACCCC

+1  Gln Gly Thr  Met Val Thr Val Ser Ser
    CCAAGGGACA ATGGTCACCG TGTCGAGT 400
    GGTTCCCTGT TACCAGTGGC ACAGCTCA
```

Figure 9C

```
+1  Glu Val Gln Leu Val Glu Ser Gly Gly Gly  Leu Val Gln Pro Gly Arg Ser
    GAGGTGCAGC TGGTGGAGTC TGGGGGAGGC TTGGTACAGC CTGGGAGGTC 50
    CTCCACGTCG ACCACCTCAG ACCCCCTCCG AACCATGTCG GACCCTCCAG

+1  _ Leu Arg Leu  Ser Cys Val Asp Ser Gly Leu Thr Phe Ser  Ser Tyr Gly Met
    CCTGAGACTC TCCTGTGTAG ACTCTGGACT CACCTTCAGT AGTTATGGCA 100
    GGACTCTGAG AGGACACATC TGAGACCTGA GTGGAAGTCA TCAATACCGT

+1  __ His Trp Val Arg Gln Ala  Pro Gly Ala Gly Leu Glu Trp Val Ala Val
    TGCACTGGGT CCGCCAGGCT CCAGGCGCGG GGCTGGAGTG GGTGGCCGTT 150
    ACGTGACCCA GGCGGTCCGA GGTCCGCGCC CCGACCTCAC CCACCGGCAA

-1  Ile Ser Tyr Asp Gly Asn Asp Lys Tyr Tyr  Ala Asp Ser Val Lys Gly Arg
    ATTTCATACG ACGGAAATGA TAAATATTAT GCAGACTCCG TGAAGGGCCG 200
    TAAAGTATGC TGCCTTTACT ATTTATAATA CGTCTGAGGC ACTTCCCGGC

+1  _ Phe Ala Ile  Ser Arg Asp Asn Ala Lys Asn Thr Leu Tyr  Leu Gln Met Asn
    ATTCGCCATC TCCAGAGACA ATGCCAAGAA CACGCTGTAT CTGCAAATGA 250
    TAAGCGGTAG AGGTCTCTGT TACGGTTCTT GTGCGACATA GACGTTTACT

+1  __ Ser Leu Thr Ile Glu Asp  Thr Ala Val Tyr Tyr Cys Ala Lys Asp Leu
    ACAGCCTGAC AATAGAGGAC ACGGCTGTCT ATTATTGTGC GAAAGATCTC 300
    TGTCGGACTG TTATCTCCTG TGCCGACAGA TAATAACACG CTTTCTAGAG

+1  Ile Glu Ser Asn Ile Ala Glu Ala Leu Trp  Gly Gln Gly Thr Leu Val Thr
    ATAGAATCAA ATATTGCGGA GGCCCTCTGG GGCCAGGGAA CCCTGGTCAC 350
    TATCTTAGTT TATAACGCCT CCGGGAGACC CCGGTCCCTT GGGACCAGTG

+1  _ Val Ser Ser
    CGTGTCGAGT                                              400
    GCACAGCTCA
```

Figure 9D

```
+1  Glu  Val  Gln  Leu  Val  Lys  Ser  Gly  Glu  Gly  Leu  Val  Lys  Pro  Gly  Gly  Ser
    GAGGTGCAGC TGGTGAAGTC TGGGGAAGGC CTGGTCAAGC CTGGGGGGTC 50
    CTCCACGTCG ACCACTTCAG ACCCCTTCCG GACCAGTTCG GACCCCCCAG

-1  Leu  Arg  Leu  Ser  Cys  Ala  Ala  Ser  Gly  Phe  Thr  Phe  Arg  Arg  Tyr  Asp  Ile
    CCTGAGACTC TCCTGTGCAG CCTCTGGATT CACCTTCAGG AGATATGATA 100
    GGACTCTGAG AGGACACGTC GGAGACCTAA GTGGAAGTCC TCTATACTAT

+1  His  Trp  Val  Arg  Gln  Thr  Pro  Gly  Lys  Gly  Leu  Glu  Trp  Val  Ser  Ser
    TCCACTGGGT CCGCCAGACT CCAGGGAAGG GCCTGGAGTG GGTCTCATCC 150
    AGGTGACCCA GGCGGTCTGA GGTCCCTTCC CGGACCTCAC CCAGAGTAGG

+1  Ile  Ser  Ser  Gly  Gly  Asn  Tyr  Ile  Asp  Tyr  Ala  Asp  Ser  Val  Lys  Gly  Arg
    ATCAGTAGTG GTGGTAATTA CATAGACTAC GCAGACTCTG TGAAGGGCCG 200
    TAGTCATCAC CACCATTAAT GTATCTGATG CGTCTGAGAC ACTTCCCGGC

+1  Phe  Thr  Ile  Ser  Arg  Asp  Asn  Ala  Asn  Asn  Val  Val  Tyr  Leu  Gln  Met  Asn
    ATTCACCATC TCCAGAGACA ACGCCAACAA TGTTGTCTAT CTACAAATGA 250
    TAAGTGGTAG AGGTCTCTGT TGCGGTTGTT ACAACAGATA GATGTTTACT

+1  Ser  Leu  Arg  Ala  Glu  Asp  Met  Ala  Val  Tyr  Phe  Cys  Ala  Arg  Asp  Gly
    ACAGCCTGAG AGCCGAGGAC ATGGCTGTCT ATTTCTGTGC GAGAGATGGG 300
    TGTCGGACTC TCGGCTCCTG TACCGACAGA TAAAGACACG CTCTCTACCC

-1  Thr  Ile  Phe  Gly  Ser  Ala  Ala  Thr  Trp  Arg  Ala  Phe  Asp  Ile  Trp  Gly  Arg
    ACGATTTTTG GATCGGCGGC GACCTGGCGG GCTTTTGATA TCTGGGGCCG 350
    TGCTAAAAAC CTAGCCGCCG CTGGACCGCC CGAAAACTAT AGACCCCGGC

+1  Gly  Thr  Met  Val  Thr  Val  Ser  Ser
    GGGGACAATG GTCACCGTGT CGAGT                              400
    CCCCTGTTAC CAGTGGCACA GCTCA
```

Figure 9E

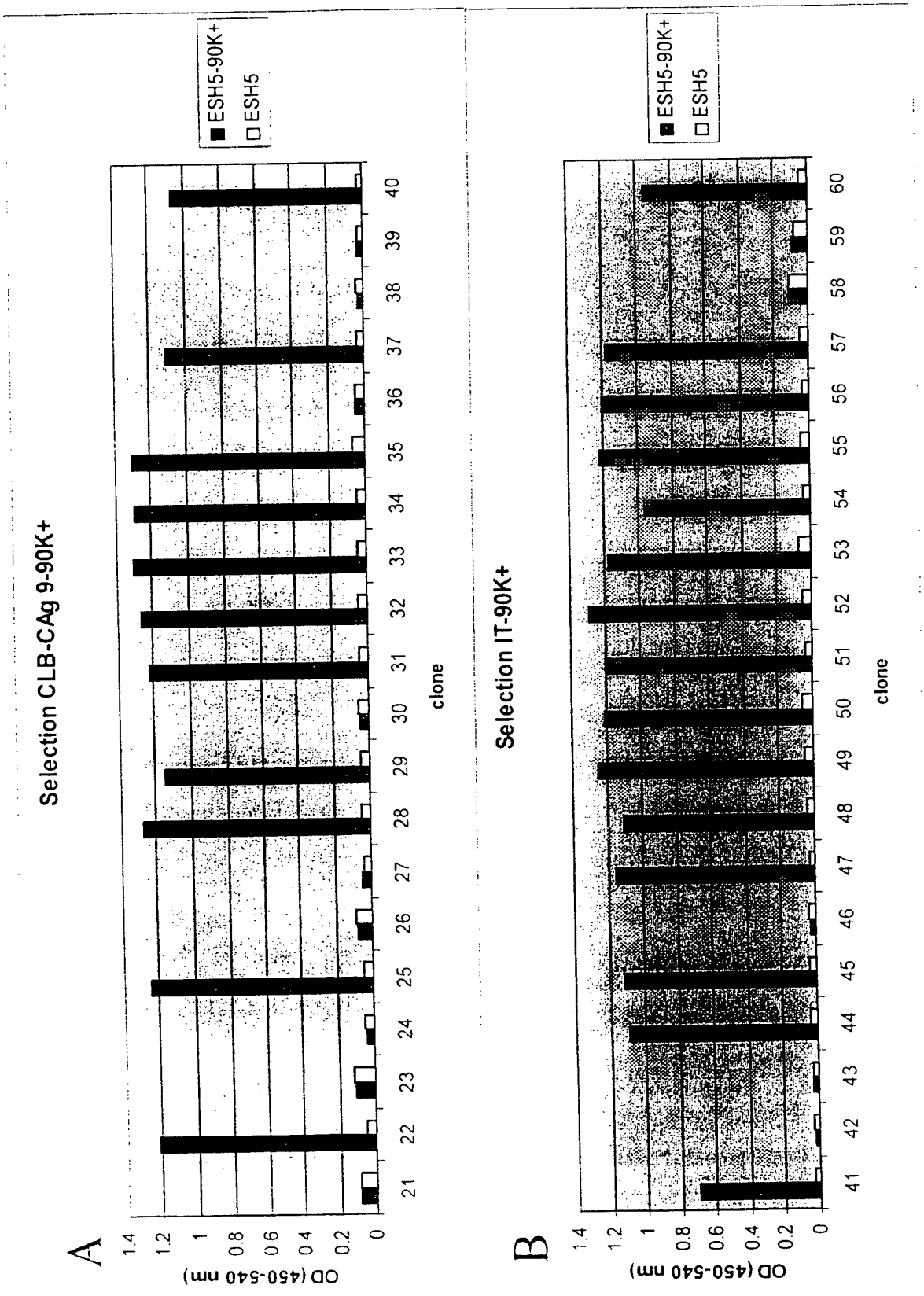
A
Selection CLB-CAg 9-90K+
ESH5-90K+
ESH5
OD (450-540 nm)
clone
B
Selection IT-90K+
ESH5-90K+
ESH5
OD (450-540 nm)
clone

Figure 10

Deduced protein sequences of isolated FVIII A2 specific scFv

Heavy chains

	FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4
	1 2 3		4	5 6	7 8 9	1 0	1 1 0 1
	123456789012345678901234567890	12345	67890123456789	012a3456789012345	67890123456789012abc345678901234	567890abcde12	34567890123
DP-10	QVQLVQSGAEVKKPGSSVKVSCKASGGTFS	SYAIS	WVRQAPGQGLEWMG	GIIPIFGTANYAQKFQG	RVTITADESTSTAYMELSSLRSEDTAVYYCAR		
	------------------------------	-H---	--------------	D----L--G--------	------------------T-T---------EL	DWFYI	WGQGTMVTVSS
DP-47	EVQLLESGGGLVQPGGSLRLSCAASGFTFS	SYAMS	WVRQAPGKGLEWVS	AISGSGGSTYYADSVKG	RFTISRDNSKNTLYLQMNSLRAEDTAVYYCAK		
	----V----D--------------------	NF---	-------------A	--G-RS-T-F-------	------------V--E----------I-----	RGRGGYKYYGMDV	WGQGTTVTVSS

Figure 11A

```
+1  Gln Val Gln Leu Val Gln Ser Gly Ala Glu  Val Lys Lys Pro Gly Ser Ser
    CAGGTGCAGC TGGTGCAGTC TGGGGCTGAG GTGAAGAAGC CTGGGTCCTC 50
    GTCCACGTCG ACCACGTCAG ACCCCGACTC CACTTCTTCG GACCCAGGAG

+1    Val Lys Val  Ser Cys Lys Ala Ser Gly Gly Thr Phe Ser  Ser His Ala Ile
    GGTGAAGGTC TCCTGCAAGG CTTCTGGAGG CACCTTCAGC AGTCATGCTA 100
    CCACTTCCAG AGGACGTTCC GAAGACCTCC GTGGAAGTCG TCAGTACGAT

+1   Ser Trp Val Arg Gln Ala  Pro Gly Gln Gly Leu Glu Trp Met Gly Asp
    TCAGCTGGGT GCGACAGGCC CCTGGACAAG GGCTTGAGTG GATGGGAGAC 150
    AGTCGACCCA CGCTGTCCGG GGACCTGTTC CCGAACTCAC CTACCCTCTG

+1  Ile Ile Pro Ile Leu Gly Thr Gly Asn Tyr  Ala Gln Lys Phe Gln Gly Arg
    ATCATCCCTA TCCTTGGTAC AGGAAACTAC GCACAGAAGT TCCAGGGCAG 200
    TAGTAGGGAT AGGAACCATG TCCTTTGATG CGTGTCTTCA AGGTCCCGTC

+1   Val Thr Ile  Thr Ala Asp Glu Ser Thr Ser Thr Ala Tyr  Met Glu Leu Ser
    AGTCACGATT ACCGCGGACG AGTCCACGAG CACAGCCTAC ATGGAGCTGA 250
    TCAGTGCTAA TGGCGCCTGC TCAGGTGCTC GTGTCGGATG TACCTCGACT

+1   Thr Leu Thr Ser Glu Asp  Thr Ala Val Tyr Tyr Cys Glu Leu Asp Trp
    GCACCCTGAC ATCTGAGGAC ACGGCCGTGT ATTACTGTGA ACTTGACTGG 300
    CGTGGGACTG TAGACTCCTG TGCCGGCACA TAATGACACT TGAACTGACC

-1  Phe Tyr Ile Trp Gly Gln Gly Thr Met Val  Thr Val Ser Ser
    TTCTATATCT GGGGCCAAGG GACAATGGTC ACCGTGTCGA GT          350
    AAGATATAGA CCCCGGTTCC CTGTTACCAG TGGCACAGCT CA
```

Figure 11B

```
+1  Glu Val Gln Leu Val Glu Ser Gly Gly Asp  Leu Val Gln Pro Gly Gly Ser
    GAGGTGCAGC TGGTGGAGTC TGGGGGAGAC TTGGTACAGC CTGGGGGGTC 50
    CTCCACGTCG ACCACCTCAG ACCCCCTCTG AACCATGTCG GACCCCCCAG

+1  Leu Arg Leu  Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser  Asn Phe Ala Met
    CCTGAGACTC TCCTGTGCAG CCTCTGGATT CACCTTTAGC AACTTTGCCA 100
    GGACTCTGAG AGGACACGTC GGAGACCTAA GTGGAAATCG TTGAAACGGT

+1  Ser Trp Val Arg Gln Ala  Pro Gly Lys Gly Leu Glu Trp Val Ala Ala
    TGAGCTGGGT CCGCCAGGCT CCCGGGAAGG GGCTGGAGTG GGTCGCGGCT 150
    ACTCGACCCA GGCGGTCCGA GGGCCCTTCC CCGACCTCAC CCAGCGCCGA

+1  Ile Gly Gly Arg Ser Gly Thr Thr Phe Tyr  Ala Asp Ser Val Lys Gly Arg
    ATTGGCGGTA GAAGTGGTAC CACATTCTAC GCGGACTCCG TGAAGGGCCG 200
    TAACCGCCAT CTTCACCATG GTGTAAGATG CGCCTGAGGC ACTTCCCGGC

+1  Phe Thr Ile  Ser Arg Asp Asn Ser Lys Asn Thr Val Tyr  Leu Glu Met Asn
    GTTCACCATC TCCAGAGACA ATTCCAAGAA CACGGTCTAT CTGGAAATGA 250
    CAAGTGGTAG AGGTCTCTGT TAAGGTTCTT GTGCCAGATA GACCTTTACT

+1  Ser Leu Arg Ala Glu Asp  Thr Ala Ile Tyr Tyr Cys Ala Lys Arg Gly
    ACAGTCTGAG AGCCGAGGAC ACAGCCATTT ATTACTGTGC GAAAAGAGGG 300
    TGTCAGACTC TCGGCTCCTG TGTCGGTAAA TAATGACACG CTTTTCTCCC

+1  Arg Gly Gly Tyr Lys Tyr Tyr Gly Met Asp  Val Trp Gly Gln Gly Thr Thr
    CGCGGGGGGT ATAAGTATTA TGGGATGGAC GTCTGGGGCC AGGGGACCAC 350
    GCGCCCCCCA TATTCATAAT ACCCTACCTG CAGACCCCGG TCCCCTGGTG

+1  Val Thr Val  Ser Ser
    GGTCACCGTG TCGAGT                                      400
    CCAGTGGCAC AGCTCA
```

Figure 11C

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(71) Applicant *(for all designated States except US)*: **STICHTING SANQUIN BLOEDVOORZIENING** [NL/NL]; Plesmanlaan 125, NL-1066 CX Amsterdam (NL).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **VOORBERG, Johannes, Jacobus** [NL/NL]; Klokkemeet 22, NL-1566 RH Assendelft (NL). **VAN DEN BRINK, Edward, Norbert** [NL/NL]; Valkenisseweg 105, NL-1069 BX Amsterdam (NL). **TURENHOUT, Ellen, Anne, Maria** [NL/NL]; Lavendel 19, NL-2211 MV Noordwijkerhout (NL).

(74) Agent: **OTTEVANGERS, S., U.**; Vereenigde, Nieuwe Parklaan 97, NL-2587 BN The Hague (NL).

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(54) Title: METHOD FOR DIAGNOSIS AND TREATMENT OF HAEMOPHILIA A PATIENTS WITH AN INHIBITOR

(57) Abstract: A polynucleotide, comprising a contiguous nucleotide sequence coding for a human antibody with factor VIII specificity, or complementary to a nucleotide sequence coding for a human antibody with factor VIII specificity, or capable of selectively hybridizing under stringent conditions to such nucleotide sequence. Such polynucleotide may be used as a probe or primer for detection of factor VIII inhibitors, or be used for producing a recombinant polypeptide. A polypeptide, comprising a contiguous amino acid sequence corresponding to or mimicking a fragment or derivative of a human antibody with factor VIII specificity capable of specific binding to factor VIII. An antibody, comprising a recombinant human antibody with factor VIII specificity or an anti-idiotypic antibody directed against a human antibody with factor VIII specificity. Pharmaceutical compositions which contain such polypeptide or antibody.